Requirement of Amino-terminal Modification for Striated Muscle α-Tropomyosin Function*

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Striated muscle α-tropomyosin expressed in Escherichia coli is unacylated, polymerizes poorly, and binds weakly to F-actin (Hitchcock-DeGregori, S. E., and Heald, R. W. (1987) J. Biol. Chem. 262, 9730-9735). To define the structural requirements of NH₂-terminal modification for striated tropomyosin function, an acetylated recombinant tropomyosin and an unacetylated short fusion recombinant tropomyosin were compared. An acetylated recombinant chicken striated muscle α-tropomyosin was expressed in insect Sf9 cells using the baculovirus expression vector system. The purified tropomyosin (~15 mg/liter of insect cell suspension) polymerized, comigrated with chicken striated α-tropomyosin purified from muscle on two-dimensional polyacrylamide gels, was blocked at the NH₂ terminus, and had the same actin affinity as muscle tropomyosin. These results conclusively show the importance of NH₂-terminal acetylation for striated tropomyosin function.

To learn if a short fusion peptide would substitute for amino-terminal acetylation, tropomyosin with AlaSerArg on the NH₂ terminus was constructed and expressed in E. coli as an unacylated protein. This β-tropomyosin bound to actin with a 10-fold higher affinity than striated muscle α-TM and, unlike muscle tropomyosin, exhibited a shear-dependent viscosity. The altered function of β-tropomyosin shows that the naturally occurring acetylated NH₂ terminus is required for full, normal function.

It is proposed that a major requirement for cooperative binding of striated muscle tropomyosin to actin is modification of the α-amino group of methionine to be an amide, as when it is acetylated or in a peptide bond in a fusion protein, to make the extreme NH₂ terminus more hydrophobic. The results are discussed in terms of known coiled coil structure.

The amino-terminal residue of most cytosolic proteins is blocked, commonly by acetylation of the α-amino group. The role of this post-translational modification is generally unknown, although it has been suggested that it may increase the half-life of proteins in cells (Bachmair et al., 1986). One of the few proteins in which NH₂-terminal acetylation has been proposed to be important for a specific function is tropomyosin (TM), an actin-binding regulatory protein found in muscle and non-muscle cells (Hitchcock-DeGregori and Heald, 1987; Zot and Potter, 1987; Lees-Miller and Helfman, 1991).

It is well known that the ends of TM are crucial for head-to-tail association as well as for F-actin binding. The amino-terminal and carboxyl-terminal nine amino acids have been postulated to associate to form an "overlap" region (McLachlan and Stewart, 1975). Removal of either the first or last 9–11 residues of striated muscle α-TM results in a large reduction in actin affinity (Ueno et al., 1976; Mak and Smillie, 1981; Dabrowska et al., 1983; Cho et al., 1990). Interestingly, striated muscle α-tropomyosin expressed in Escherichia coli polymerizes poorly and binds weakly to F-actin compared with TM isolated from muscle (Hitchcock-DeGregori and Heald, 1987; Heald and Hitchcock-DeGregori, 1988; Cho et al., 1990; Willadsen et al., 1992). The only known difference between the muscle and E. coli-expressed protein is the absence of NH₂-terminal acetylation in the E. coli protein.

To establish the importance of NH₂-terminal acetylation for striated muscle α-TM function, and that the defect of the E. coli-expressed protein is not a consequence of some other difference between recombinant and naturally occurring TM, we have expressed a fully functional acetylated chicken striated muscle α-TM in insect cells using the baculovirus expression vector system. In addition, we have explored the sufficiency of another NH₂-terminal modification of α-TM for actin binding and head-to-tail polymerization. We propose a major requirement for high affinity cooperative binding of striated muscle α-TM to actin is modification of the α-amino group of met 1 to be an amide, as when it is acetylated or in a peptide bond in a fusion protein. However, only N-acetylated TM has normal actin affinity and polymerizability showing the importance of the authentic NH₂-terminal modification for function. Portions of this work have been reported in a preliminary form (Urbanickova and Hitchcock-DeGregori, 1993a, 1993b).

MATERIALS AND METHODS

Cloning and Expression of Tropomyosin cDNA Using the Baculovirus Expression Vector System—For expression of a chicken striated muscle α-tropomyosin (α-TM) in Sf9 insect cells, we cloned an α-TM cDNA (Gooding et al. (1987), gift of S. MacLeod) into the non-fusion transfer vector pBlueBac I (Invitrogen). Nhel sites were created in the α-TM cDNA (cloned in M13 mp18 at the EcoRI site) by ligating Nhel linkers (GGCTAGCC) to the filled in NcoI site at the 5′ end of the coding region, and to the HinClI site in the M13 polylinker at the 3′ end of the cDNA. The Nhel insert containing the α-TM cDNA was cloned into the unique Nhel site in pBlueBac I downstream from the polyhedrin promoter. The

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1 The abbreviations used are: TM, tropomyosin; BEVS, baculovirus expression vector system; BEVS-TM, tropomyosin expressed in Sf9 cells; α-TM, tropomyosin expressed in E. coli with a 3-residue NH₂-terminal fusion peptide; NpelHGE, non-equilibrium pH gel electrophoresis; PAGE, polyacrylamide gel electrophoresis; Tn, tropinin; Sf9, Spodoptera frugiperda insect cells; AcNPV, Autographa california nucleopolyhedrosis virus.
sequence of the 5′ end of the cDNA in pBlueBac is:

\[
\text{NheI} \quad \text{NcoI} \\
\text{GGC TAG CCC ATG GAT} \\
\text{Met Asp}
\]

(Spotted) frugiperda (Sf9) insect cells and Autographa californica nuclear polyhedrosis virus (AcNPV) were purchased from Invitrogen as a kit. Cells were cultured at 27 °C in Grace's insect medium (Life Technologies, Inc.) supplemented with 0.33% TC Yeastolate (Difco), 0.33% lactalbumin hydrolysate (Sigma), 10% fetal calf serum (Life Technologies, Inc.), and 50 μg/ml gentamicin (Sigma), and 2.5 μg/ml amphotericin B (Summers and Smith, 1987). The medium for cells in suspension culture was supplemented with 0.1% Pluronic F-68 (Sigma).

Sf9 cells were cotransfected with the recombinant transfer vector pBlueBac and a kit. Cells were cultured at 27 °C in Grace's insect medium (Life Technologies, Inc.) supplemented with 0.1% Pluronic F-68 (Sigma).

Cells were harvested 48-50 h post infection for purification of recombinant TM.

Construction and Expression of β-Tropomyosin—To create a TM with a short NH2-terminal fusion peptide, the 5′ end of the chicken α-TM cDNA (Gooding et al., 1987) was modified for cloning into the NheI and BamHI sites in pET11b (Studier et al., 1990). The NcoI site at the 5′ end of the TM cDNA (in M13mp18) was digested and filled in using the Klenow fragment of E. coli DNA polymerase I. NheI linkers (CGGGCTAGCGCGCAAGCTTCG) were ligated to the resulting blunt end. The NheI-BamHI fragment containing the cDNA was cloned into pET11b and was transformed into BL21(DE3) for expression. The resulting cDNA encodes a full-length TM with additional amino acids on the NH2-terminal end. Since the second amino acid in Ala, the NH2-terminal Met should be removed by E. coli, as was the case (see Equation 2). Hence, the fusion protein is called βTM.

\[
\text{NheI} \\
\text{ATG GCT AGC CGC ATG} \\
\text{Met Ala Ser Arg Met}...
\]

Protein Purification—For preparation of BEVS-TM, Sf9 cells were harvested by centrifugation and washed as recommended by Invitrogen. Cells were lysed in an extraction buffer (50 mM NaCl, 50 mM imidazole, pH 7.0, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, 0.1 mM leupeptin, 0.1 mM chymostatin). The lysate was centrifuged at 9000 rpm (Servall BC-5B rotor SS-34) for 20 min at 4 °C. The extraction was repeated once, and the supernatants were combined.

BEVS-TM was purified using heat denaturation, ammonium sulfate fractionation, anion exchange chromatography on DE52 cellulose (Whatman), and hydroxyapatite chromatography (Bio-Gel HT, Bio-Rad) as described by Hitchcock-DeGregori and Heald (1987) except that heat denaturation was for 10 min and 0.5 mM EDTA was included in the buffer for the DE52 column. Fractions with TM were identified using SDS-polyacrylamide gel electrophoresis and were dialyzed against 50 mM NH4HCO3 and lyophilized. βTM was expressed in E. coli BL21(DE3) (Sudier et al., 1990) and purified as described previously (Hitchcock-DeGregori and Heald, 1987).

NH2-terminal Sequence Determination—The amino acid sequences of the first 10–13 residues of BEVS-TM, unacylated (bacterial) TM, and βTM were determined at the W. M. Keck Foundation Resource Laboratory at Yale University on an Applied Biosystems, Inc. model 470A or 477A instrument with a model 120a phenylthiohydantoin-derivative analyzer. The high-performance liquid chromatography system was modified as described by Tempst et al. (1989).

The sequence of the first 10 residues of unacylated TM was Met-Asp-Ala-Ile-Lys-Lys-Lys-Met-Gln-Met, identical to that of chicken striated a-TM encoded by the cDNA (Gooding et al., 1987). The first cycle yield was 692 pmol, and the repetitive yield was 89.5%. The initial first cycle sequencing yield was 79.6% based on amino acid analysis, indicating that virtually all of the NH2-terminal methionine was unblocked. The fusion TM cDNA encoded four additional residues at the NH2 terminus: MetAlaSerArg, followed by the full TM sequence. Sequence determination of the first 10 residues showed that the first four residues were AlaSerArg, followed by the TM sequence (Met-Asp-Ala-Ile-Lys-Lys-Lys-Met-Gln-Met). Thus, the NH2-terminal Met was removed by E. coli, as anticipated. The first cycle yield was 132 pmol with a repetitive yield of 91.7%.

The sequence determination of the BEVS-TM showed no full-length TM sequence indicating that the NH2 terminus of TM expressed in Sf9 cells was blocked. The sample analyzed was that in Fig. 3a which contains multiple components. The major sequences in the analysis were TM lacking the first 11 or 12 residues of TM (<Lys-Leu-Aep-Lys-Glu-Asn-Ala-Leu-Aep-Arg-Aa-Glu-Gln-Ala>). The first cycle yield was 58 pmol, and the repetitive yield was 94.7%.

Purification of BEVS-TM by Prebinding to Actin—To purify BEVS-TM that preferentially bound to F-actin, we followed the procedure of Pittenger and Heilmann (1992) with modifications. BEVS-TM was combined with F-actin (1 mg/ml) and incubated at room temperature for 1 h in 150 mM NaCl, 20 mM imidazole pH 7.0, 2 mM MgCl2, 0.5 mM dithiothreitol. The mixture was centrifuged at 60,000 rpm in a Beckman model TL-100 centrifuge (TLA 100 rotor for 24 h). The pellet was resuspended in the same buffer and the NaCl concentration was increased to 600 mM to dissociate the TM from actin. The sample was warmed at 65 °C for 10 min and the denatured actin was separated by centrifugation. Tropomyosin was precipitated at its isoelectric point from the supernatant, denatured, and the pellet was resuspended in the above buffer and dialyzed against it.

Actin Binding Assay—The binding affinity of TM to F-actin was measured by cosedimentation as described previously (Hitchcock-DeGregori and Heald, 1987) with modifications. Centrifugation was carried out at 60,000 rpm for 25 min at 20 °C in a Beckman model TL-100 centrifuge (TLA 100 rotor). The pellets and supernatants were analyzed on 12% SDS-polyacrylamide gels (Laemmli, 1970) stained with Coomassie Blue. The amount of TM that sedimented in the absence of actin was negligible. Quantification of bound and free TM was carried out using a Molecular Dynamics model 300A computing densitometer. The concentration of free TM in the supernatant was determined using chicken pectoral muscle α-TM as a standard. An internal BSA standard included in the sample buffer allowed for corrections due to variations in sample loading. Binding constant (Kb) and Hill coefficient (αH) were calculated using SigmaPlot version 5.0 program to fit the following equation to the data:

\[
v = n[TM]^{αH} + [TM] = K_{D} - [TM]
\]

Viscosity Measurements—Viscosity experiments were carried out at 25 °C in a Cannon 100 viscometer using a 1 ml volume. Typical outflow time of buffer alone was about 70 s. Tropomin and TM solutions were dialyzed against 10 mM imidazole, pH 7.0, 20 mM NaCl, 2.0 mM MgCl2, 0.5 mM dithiothreitol and centrifuged before analysis.

Gel Electrophoresis and Immunoblotting—One- and two-dimensional polyacrylamide gel electrophoresis (12%) were performed according to Laemmli (1970) and OFarrell (1975). For isoelectric focusing, ampholytes Bio-Lyte range 4-6 (Bio-Rad) were used.

Immuno blotting was carried out as described by Towbin et al. (1979) using a Vectastain ABC kit (Vector Laboratories). Nitrocellulose sheets with transferred proteins were probed with affinity purified goat anti chicken striated muscle α-tropomyosin.

General Methods—Routine recombinant DNA methods such as transformation, agarose and polyacrylamide gel electrophoresis, small and large scale preparation of plasmids, isolation of fragments from agarose gels, phosphorylation, dephosphorylation, ligation and enzyme digestion were carried out according to Sambrook et al. (1989) as recommended by the supplier.

Muscle proteins used in the assays were purified following published preparations of the chicken pectoral muscle α-TM (Hitchcock-DeGregori et al., 1985), unacylated TM produced in E. coli (Rogers and Heald, 1987), chicken pectoral muscle actin from acetone powder (Hitchcock-DeGregori et al., 1982), chicken pectoral muscle tropomin (Potter, 1982). Protein concentration was determined spectrophotometrically using the extinction coefficients (1% at 280 nm): actin, 11.0; TM, 2.8; troponin, 4.5. The concentration of the TM used for the standard curve for the densitometry was determined using a microbiuret assay (Goa, 1954).
Expression and Purification of BEVS-Tropomyosin—The expression and purification of α-TM in Sf9 cells was monitored by immunoblot analysis of SDS-polyacrylamide gels using affinity-purified goat polyclonal antibody to chicken striated muscle α-TM (Fig. 1). There was no detectable TM in uninfected Sf9 cells or in Sf9 cells infected with wild type baculovirus indicating that the antibody does not cross-react with an endogenous TM \( \text{(lanes 1 and 2)} \). After infection with the recombinant virus the major protein band cross-reacted with anti-TM \( \text{(lane 3)} \). The BEVS-TM was soluble and heat-stable and could be purified using conventional methods \( \text{(lanes 4–6)} \) to yield about 12–18 mg/liter of cell suspension. Optimal TM expression was at 2 days post infection. At that time only a small amount of TM was detected in the medium, presumably due to lysed cells \( \text{(lane 7)} \). After longer times the amount of TM in the medium increased.

Actin Affinity of BEVS-TM—Fig. 2a shows that the BEVS-TM bound to actin with a \( K_{\text{app}} \) of \( 3.8 \times 10^{6} \text{ M}^{-1} \) \( (n=3) \), slightly lower than that of muscle α-TM \( (K_{\text{app}} = 4.8 \times 10^{6} \text{ M}^{-1}, n=3) \). The affinity is much higher than that of unacetylated TM expressed in \( E. \text{coli} \) which is too weak to measure, as previously reported (Heald and Hitchcock-DeGregori, 1988; Cho and Hitchcock-DeGregori, 1991; Willadsen et al., 1992). When BEVS-TM was further purified by prebinding to F-actin, the actin affinity was indistinguishable from that of muscle TM (Fig. 2b).

We were initially concerned that the slightly lower actin affinity of BEVS-TM was due to incomplete NH\(_2\)-terminal acetylation since the baculovirus expression vector system is known to result in different efficiencies of posttranslational modifications of overexpressed proteins (O’Reilly et al., 1992). This explanation is unlikely since no complete NH\(_2\)-terminal amino acid sequence was obtained. Two-dimensional PAGE, however, indicated heterogeneity. The most basic form of BEVS-TM comigrated with muscle TM (compare Fig. 3, b and d), had a more acidic isoelectric point than unacetylated TM, and selectively bound to F-actin (Fig. 3c and 4a).

The more acidic forms in the heterogeneous BEVS-TM preparation shown in Fig. 3a are TMs that are missing the first 11 or 12 residues (see the NH\(_2\)-terminal sequence determination in "Materials and Methods"). Based on our previous analysis of a NH\(_2\)-terminal deletion mutant, the truncated forms would not be expected to bind to actin (Cho et al., 1990). The acidic forms are not a consequence of phosphorylation since treatment of BEVS-TM with alkaline phosphatase (Heeley et al., 1989) did not reduce their amounts. The quite homogeneous preparation shown in Fig. 4c was used for measuring actin affinity in Fig. 2a.

Troponin (Tn) increased the affinity of both muscle TM and BEVS-TM to \( > 10^{6} \text{ M}^{-1} \), more than 10-fold, too tight to measure accurately. Together these results show that the chicken striated muscle α-TM expressed in Sf9 cells is indistinguishable
from muscle TM in these assays. To our knowledge, the only difference between these TMs and the recombinant TM expressed in E. coli is the presence of an acetylated NH2-terminal methionine. The NH2-terminal and COOH-terminal sequences of the unacetylated protein are as predicted by the cDNA sequence (Hitchcock-DeGregori and Heald, 1987; Cho et al., 1990; this work). Also, it migrates as a single species in two-dimensional PAGE, one charge more basic than acetylated chicken striated muscle α-TM purified from muscle or overexpressed in Sf9 cells, as expected. Modifications such as acetylation, phosphorylation, deamidation, and multiple methylations all change the isoelectric point of the protein. While it is possible that some other modification has gone undetected, we have every reason to conclude that the only difference between muscle TM and TM expressed in E. coli is the state of NH2-terminal acetylation, conclusively showing the importance of postranslational modification for striated α-TM function.

**FIG. 3. Analysis of different TMs by two-dimensional PAGE.** a, a partially purified preparation of BEVS-TM; b, mixture of muscle TM and BEVS-TM prebound to actin; c, chicken striated muscle α-TM; d, chicken striated muscle α-TM. The most basic form in BEVS-TM (shown with arrowheads) comigrates with muscle TM and selectively binds to actin. The more acidic forms are the proteolytic products, which are unacetylated and protonated, would be one and two charges, respectively, more negative than full-length TM. The high pH side of the gel is at the right side of each panel.

**FIG. 4. Pre-binding of BEVS-TM to actin.** Analysis by two-dimensional PAGE. a, BEVS-TM that prebound to actin; b, unbound material = supernatant after prebinding the BEVS-TM shown in Fig. 2b to actin. The more acidic forms migrated slightly faster than full-length TM in the SDS dimension of the gel, consistent with being truncated; c, a purer BEVS-TM preparation that was used in Fig. 2a and Table I. The arrowheads indicate the most basic form of BEVS-TM that comigrates with muscle TM. The high pH side of the gel is at the right side of the panel.

**FIG. 5. Actin binding of chicken striated muscle α-TM (○) and β3TM (●).** The experiment was carried out and data analyzed as described in Fig. 2a except the data were not normalized. Data from a single experiment are shown. The data for chicken striated muscle α-TM are the same as for Fig. 2a.

**Actin Affinity of a Short Fusion Tropomyosin.—** We have been interested in the mechanism by which N-acetylation of striated α-TM increases actin affinity. N-Acetylation of TM and of an NH2-terminal TM peptide stabilizes the coiled coil conformation. We have proposed that this is primarily a consequence of interchain hydrophobic interactions between the acetyl group of one chain and the methionine of the opposite chain (Greenfield et al., 1994). The resulting structure may be crucial for cooperative actin binding.

Tropomyosins with long NH2-terminal fusion peptides (35 or 80 residues) have the same or slightly higher affinity for actin than muscle TM (Heald and Hitchcock-DeGregori, 1988; Stone and Mendelson, 1989). Possibly fusion TMs bind to actin because the fusion peptide introduces an amide bond to the α-amino group of the NH2-terminal Met, as does N-acetylation. To test the hypothesis, we designed a fusion TM with only three additional residues at the NH2 terminus, β3TM (Ala-Ser-Arg-TM), and expressed it in E. coli (with an unacetylated NH2-terminal Ala). While we had predicted that β3-TM would bind to actin, we were surprised that its actin affinity was more than 10-fold higher than muscle TM (Fig. 5). β3TM does not sediment in the absence of F-actin.

**Polymerization of BEVS-TM and β3TM—** It is well established that modifications at the ends of striated TM alter polymerizability at low ionic strength (Ueno et al., 1976; Johnson and Smillie, 1977; Hitchcock-DeGregori and Heald, 1987; Heeley et al., 1989; Stone and Mendelson, 1989; Heeley, 1994). We previously reported that unacetylated TMs polymerize poorly compared to muscle TM (Hitchcock-DeGregori and Heald, 1987; Cho and Hitchcock-DeGregori, 1991). BEVS-TM polymerized better than unacetylated TM but its viscosity was lower than that of muscle TM (Table I). Troponin increased the viscosity. The lower viscosity of BEVS-TM compared to muscle TM is due to the presence of non-polymerizable products in the preparation (Fig. 4c), an effect that is magnified because viscosity is not linearly related to concentration, as is clear in Table I. It is not practical to measure the high shear viscosity of BEVS-TM purified by prebinding to actin. While heating irreversibly reduces the viscosity of smooth muscle TM (Graceffa, 1992), it is unlikely that heat denaturation, a step in BEVS-TM purification, is primarily responsible for the lower viscosity. Heating, followed by cooling, of striated muscle α-TM resulted in only a 25% reduction in viscosity (results not shown).

β3TM had a much higher viscosity than muscle α-TM and it increased in the presence of Tn (Table I, Fig. 6). The behavior
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**Table I**

Viscosity measurements of tropomyosins

| Tropomyosin          | Muscle TM | Unacetylated TM | BEVS-TM | fSTM* |
|----------------------|-----------|----------------|---------|-------|
| Tropomyosin, 1.0 mg/ml | 0.48      | 0.082          | 0.20    | 2.7   |
| Tropomyosin, 0.5 mg/ml | 0.15      | 0.069          | 0.070   | 0.29  |
| $\eta_0$             | 0.83      | 0.13           | 0.26    | 0.48  |
| $\eta_a$             |           |                |         |       |

* The measurement for fSTM at 1 mg/ml was taken after dialysis but without freezing. The measurements with and without Tn were at concentrations of 0.33 mg/ml fSTM, 0.53 mg/ml Tn. The sample had been frozen and thawed twice after dialysis.

Fig. 6. Specific viscosity of muscle $\alpha$-TM (●) and fSTM (○) as a function of number of measurements. Conditions: 10 mM imidazole, pH 7.0, 20 mM NaCl, 2.0 mM MgCl$_2$, 0.5 mM dithiothreitol, $T = 25.4$ °C.

**DISCUSSION**

In this study we have investigated the effect of NH$_2$-terminal modifications on TM function. We have shown that NH$_2$-terminal acetylation is important for cooperative binding to actin and head-to-tail polymerization of striated muscle $\alpha$-TM. Although this conclusion was inferred from studies of unacetylated recombinant TMs expressed in E. coli, the expression of a fully functional recombinant chicken striated muscle $\alpha$-tropomyosin with a blocked amino terminus in insect SF9 cells establishes the point. There had been concern that the altered function of unacetylated recombinant TM may have been a consequence of differences other than the unacetylated NH$_2$ terminus, but there is no evidence for additional differences. Moreover, it will now be possible to investigate the importance of individual residues in the conserved NH$_2$-terminal sequence for TM function. The main advantages of expression in insect SF9 cells over vertebrate expression systems are the high expression level and the absence of closely related cellular TMs that may interfere with our analyses. A disadvantage of the baculovirus expression vector system is the presence of persistent proteases, a problem that has been addressed by improving the purification method. Tropomyosin expressed in E. coli with a three residue NH$_2$-terminal fusion peptide is significantly different from acetylated TM in actin affinity, polymerizability, and thermal stability, limiting its usefulness as an analogue.

We have previously shown that NH$_2$-terminal acetylation can stabilize the coiled-coil $\alpha$-helix of a 32-residue NH$_2$-terminal TM peptide as well as full-length TM (Greenfield et al., 1994). While the structure of the ends of TM is unknown (Phillipe et al., 1986; Whitby et al., 1992), modeling of the peptide based on its homology with the GCN4 leucine zipper (O'Shea et al., 1991) has shown that the NH$_2$-terminal acetyl groups may participate in inter-chain hydrophobic interactions (Greenfield et al., 1994). The model is consistent with the GCN4 structure in which an amino acid in an $a$ position in the coiled coil (corresponding to Met-1) interacts with the preceding $g$ residue (an Arg) on the opposite chain (corresponding to the acetyl group).

Study of NH$_2$-terminal fusion TMs has given further insight into the structural significance of N-acetylation. Our studies and those of others have shown that fusion TMs with 2 (the shortest) to 80 (the longest) residues all bind well to actin (Hitchcock-DeGregori and Heald, 1987; Heald and Hitchcock-DeGregori, 1988; Stone and Mendelson, 1989; Montiero et al., 1994; this work). Fusion TMs with 11 or fewer residues polymerize at low ionic strength. Comparison of the sequences of these fusion peptides clearly shows that the high actin affinity is indifferent to the sequence, though this has not been systematically investigated. The 3 residues of the fusion peptides closest to TM's own initiating Met are Lys-Met-Thr (Hitchcock-DeGregori and Heald, 1987; Heald and Hitchcock-DeGregori, 1988), Glu-Gly-Arg (Stone and Mendelson, 1989; Montiero et al., 1994), Ala-Ser (this work), Ala-Ala-Ser (Montiero et al., 1994). Even though certain of these fusion TMs are similar in function to striated muscle TM, none is identical, making BEVS-TM preferable for detailed analysis of the functional significance of NH$_2$-terminal residues. The NH$_2$-terminal sequence is highly conserved throughout phylogeny in 284 residue TMs, and it would be surprising if any modification was without effect.

We postulate that a major requirement for high affinity cooperative binding of striated muscle $\alpha$-TM to actin is modification of the $\alpha$-amino group of Met-1 to be an amide, as when it is acetylated or in a peptide bond in a fusion protein. As a consequence, the positive charge of the initial Met is removed and the extreme NH$_2$ terminus would be more hydrophobic, allowing interchain interactions as described in the GCN4 leucine zipper peptide structure (O'Shea et al., 1991). In fSTM, the $n$-1 residue, an Arg, is in a $g$ position, comparable to the sequence of the GCN4 leucine zipper peptide. Merely removing the positive charge at the NH$_2$ terminus by deprotonating the
\(\alpha\)-amino group at high pH, however, is insufficient for actin binding and NH\(_2\)-terminal stabilization although the interpretation of the results may not be so straightforward (Cho et al., 1990; Greenfield et al., 1994).

Interestingly, the presence of a positively charged Arg in the \(n\)-1 position of f\(\text{TM}\) does not have the same detrimental effect on actin affinity as the \(\alpha\)-amino group of unacylated TM's own Met. The positive charge on the Arg would be removed from the peptide backbone due to the long hydrophobic side chain and therefore would not be subject to interchain electrostatic repulsion. A negatively polar residue at the \(n\) position (Thr, Ser) also does not inhibit actin binding. It is unlikely that the modifications at the NH\(_2\) terminus have their major effect on function as a consequence of stabilizing the helix dipole by removing the positively charged \(\alpha\)-amino group from the interface \(\alpha\) position. First, the f\(\text{TM}\) has the same stability to thermal denaturation as the unacylated protein. Second, the stabilization of a 32 residue NH\(_2\)-terminal TM peptide by N-acetylation cannot be explained by interactions with the helix dipole (Greenfield et al., 1994).

The polymerizability (high viscosity at low ionic strength) of the shorter fusion TM is difficult to evaluate. The shear dependence of the viscosity of f\(\text{TM}\) indicates that the naturally occurring acetylated NH\(_2\)-terminal sequence is required for the stable polymerization seen with muscle TM. Nevertheless, TN causes an increase in the viscosity of f\(\text{TM}\) (Table I) even though it has only a small effect on unacylated TM or the 80 residue fusion TM (Hitchcock-DeGregori and Heald, 1987; Cho and Hitchcock-DeGregori, 1991). The shear dependence of the viscosity of other fusion TMs was not evaluated (Monteiro et al., 1994).

It has fascinated us that the lack of acetylation of the NH\(_2\)-terminal Met of striated muscle \(\alpha\)-TM has a greater effect on TM function than more extensive modifications of the COOH terminus. The properties of unacylated TM are similar to those of TM lacking the last eleven residues (Hitchcock-DeGregori and Heald, 1987; Butters et al., 1993), whereas removal of just the last 3 residues of TM has little effect on function (Ueno et al., 1976; Johnson and Smillie, 1977). Furthermore, the two ends of TM act cooperatively to result in high actin affinity. Carboxypeptidase digestion of unacylated striated muscle \(\alpha\)-TM does not further weaken actin affinity (Butters et al., 1993) and NH\(_2\)-terminal acetylation is not so crucial for the actin binding of TMs containing the smooth \(\alpha\)-TM ninth exon (Cho and Hitchcock-DeGregori, 1991; Pittenger and Helfman, 1992; Novy et al., 1993). Calcium-dependent regulation of the actomyosin ATPase with TN is relatively insensitive to N-acetylation (Hitchcock-DeGregori and Heald, 1987; Cho and Hitchcock-DeGregori, 1991).

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