

Functional Homodimers and Heterodimers of Recombinant Smooth Muscle Tropomyosin[†]

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ABSTRACT: Skeletal and smooth muscle tropomyosin (Tm) require acetylation of their N-termini to bind strongly to actin. Tm containing an N-terminal alanine–serine (AS) extension to mimic acetylation has been widely used to increase binding. The current study investigates the ability of an N-terminal AS extension to mimic native acetylation for both $\alpha\alpha$ and $\beta\beta$ smooth Tm homodimers. We show that (1) AS α -Tm binds actin 100-fold tighter than α -Tm and 2-fold tighter than native smooth $\alpha\beta$ -Tm, (2) β -Tm requires an AS extension to bind actin, and (3) AS β -Tm binds actin 10-fold weaker than AS α -Tm. Tm is present in smooth muscle tissues as >95% heterodimer; therefore, we studied the binding of recombinant $\alpha\beta$ heterodimers with different AS extensions. This study shows that recombinant Tm requires an AS extension on both α and β chains to bind like native Tm and that the α chain contributes more to actin binding than the β chain. Once assembled onto an actin filament, all smooth muscle Tm's regulate S1 binding to actin Tm in the same way, irrespective of the presence of an AS extension.

Tropomyosin (Tm) is an actin binding, α -helical, coiled-coil protein dimer which binds along the length of actin filaments in both muscle and nonmuscle cells and thus cooperatively regulates the interaction of actin with myosin heads. Muscle cells contain Tm expressed via two genes, Tm1 and Tm2 (α and β), and isoform diversity (smooth and skeletal) results from the alternative splicing of the two genes. Each monomer of the dimer coiled coil contains 284 amino acid residues (1–3).

In skeletal muscle tissue Tm is present predominantly as a mixture of $\alpha\beta$ heterodimer and $\alpha\alpha$ homodimer (4–6). $\beta\beta$ is less stable and is much less common in muscle tissues (5, 6). Studies involving guanidine hydrochloride dissociation of native homodimer chains and anion-exchange chromatography have shown that smooth muscle contains roughly equal amounts of α and β chains, more than 95% of which is present as heterodimer (7–9). Thermal unfolding studies also show that the heterodimer is preferentially formed at temperatures below its unfolding transition (9). Preference of the heterodimer is not fully understood; however, viscosity and thermal unfolding measurements indicate that the strength of the end-to-end interaction of the smooth β heterodimer is much higher than that of the $\alpha\alpha$ and $\beta\beta$ homodimers which have about the same strength of end-to-end interaction (7, 8, 10). The strength of end-to-end interactions in the heterodimer might affect the cooperative behavior of the tropomyosin and thus might provide a functional advantage for the predominance of heterodimer

in native smooth muscle. The ability to be able to assemble in vitro heterodimers would be very valuable. Such ability could be used to monitor the behavior of a single α or β chain on the dimer via either mutation or labeling. The current study investigates the properties of heterologously expressed and assembled heterodimers, including actin binding and the regulation of S1¹ binding to actin.

Skeletal and smooth muscle Tm requires acetylation of its N-terminus in order to bind strongly to actin and thus regulate muscle contraction (11, 12). The precise mechanism by which this acetylation mediates actin binding is not fully understood, but it is known that the end-to-end Tm–Tm contacts are essential for the process and that these contacts are stronger in smTm than in skTm. Recent structural data for the striated Tm have shed more light on the nature of the overlap region between neighboring Tm's. The coiled coil at the C-terminal region of the dimer is splayed approximately over the last 10 amino acids, while the N-terminus maintains its coiled-coil structure, provided the N-terminus is acetylated (13, 14). This allows the N-terminus to “slot” inside the C-terminus, thus enabling the polymerization of Tm into filaments (15). The presence of an acetyl group at the N-terminus or an extension to the sequence has been shown to be essential for maintaining the coiled-coil structure in this region (11).

Expression of recombinant Tm in the bacteria *Escherichia coli* is easily undertaken and has the advantage of obtaining

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¹ Abbreviations: AS, N-terminal alanine–serine extension; AS α -Tm, α -Tm, alpha smooth tropomyosin; AS β -Tm, β -Tm, beta smooth tropomyosin; S1, myosin subfragment 1; EGTA, ethylene glycol bis-(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; chk β Tm, chicken beta smooth tropomyosin.

high yields, quickly. The alternative is expression in insect cells which can produce expression levels of 120–200 mg/L of infected cells using special enhancer sequences (16). *E. coli* cells are prokaryotic and thus generally do not acetylate the N-terminus of eukaryotic proteins although there are exceptions (17). Tm expressed in *E. coli* is not acetylated and will be largely inactive (14, 18). Tm containing an N-terminal alanine–serine (AS) extension to mimic acetylation has been devised and shown to restore activity for skeletal Tm (19, 20). The current study is designed to investigate the efficacy of the N-terminal AS extension as a mimic of native acetylation for both α - and β -smTm. This study identifies the importance of acetylation and the AS extension in smooth muscle Tm/actin binding and the regulation of S1 binding to actin via the comparison of the bacterially expressed AS-smTm's with naturally acetylated Tm from chicken gizzard.

EXPERIMENTAL PROCEDURES

Construction of Vectors for Expression of Tm. Plasmid vectors for the expression of chicken gizzard AS α -Tm, and α -Tm, were constructed as described in ref 21. AS β -Tm and β -Tm were constructed by cloning *NdeI/BamHI* purified, digested PCR-amplified fragments into the *NdeI/BamHI* restriction sites of the pJC20 expression vector. PCR was carried out using the pAED4-91 vector construct as the template. AS α -Tm and AS β -Tm contain the chicken gizzard α -tropomyosin and β -tropomyosin genes cloned in pJC20 and express Tm containing an N-terminal Met-Ala-Ser (ASTm). The N-terminal Met is removed posttranslationally. α -Tm and β -Tm contain the chicken gizzard α -tropomyosin and β -tropomyosin genes cloned in pJC20 and express. DNA was sequenced to ensure fidelity of the PCR amplification. The following nucleotides were used in the PCR reactions for the construction of AS β -Tm and β -Tm: AS-forward chk β Tm, 5' GGA ATT CCA TAT GGC GAG CAT GGA GGC CAT CAA GAA GAA GAT GC 3'; forward chk β Tm, 5' GGA ATT CCA TAT GGA GGC CAT CAA GAA GAA GAT GC 3'; reverse chk β Tm, 5' CGC GGA TCC TCA GAG GTT GTT CAG CTC CAG CAA GGT CTG G 3'.

Preparation of Proteins. Native chicken gizzard tropomyosin was prepared as described previously (22). Tm was further purified by ammonium sulfate fractionation and anion-exchange chromatography on DEAE-cellulose.

Recombinant AS α -Tm, α Tm, AS β -Tm, and β -Tm were expressed in bacteria (strain BL21 DE3). One liter cultures were grown to exponential phase and induced for 3 h with 100 mg/L IPTG. Cells were harvested and resuspended in 30 mL of cold lysis buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EGTA, and 5 mM MgCl₂) and lysed by sonication. The majority of *E. coli* proteins were precipitated by heating to 80 °C for 10 min. The precipitated protein and cell debris were then removed by centrifugation. The soluble Tm was then isoelectrically precipitated at pH 4.5. The precipitate was pelleted and resuspended in 10–20 mL (dependent upon yield) of running buffer (10 mM PO₄³⁻, pH 7.0, 100 mM NaCl) with 10 mg/L DNase and 10 mg/L RNase and incubated at 4 °C for 2 h. The Tm was then further purified using 2 × 5 mL Pharmacia HiTrap-Q columns in tandem, eluted with a 100–900 mM NaCl gradient, the Tm eluting at ~250–350 mM NaCl. Fractions

were analyzed by SDS–PAGE, pooled, and concentrated by isoelectric precipitation. Protein concentrations were determined by their absorbance at 280 nm using a molar extinction coefficient $E_{280\text{nm}}$ of 8400 M⁻¹ cm⁻¹ for α -Tm, and 11200 M⁻¹ cm⁻¹ for β -Tm, for the dimer in 5 mM Tris-HCl buffer, pH 7.0, respectively.

Preparation of Tm Heterodimers. α/β -Tm heterodimers were prepared using the method described in ref 8. Separate samples of α -Tm and β -Tm homodimers were heated to 65 °C in the presence of 20 mM DTT to ensure reduction of the cysteine side chains (C190 in α -Tm and C36 in β -Tm) in a buffer of 20 mM MOPS, 500 mM KCl, and 5 mM MgCl₂, pH 7.0. Equimolar quantities of homodimers were then mixed at 65 °C, allowed to cool to 37 °C, and incubated for ~2 h to allow dimer formation. For smTm it has been shown that the heterodimer is preferentially assembled (9, 10), and this can be confirmed by examining the ability to form Cys–Cys cross-links between the two chains of Tm. α - and β -Tm each have a single Cys (Cys-190 in α -Tm and Cys-36 in β -Tm), and under oxidizing conditions interchain cross-links will be formed in the homodimers. For the $\alpha\beta$ heterodimer the two cysteines are remote from each other, and no cross-links will form. A sample of the Tm mixture was used in the assay; DTT was removed via a Bio-Rad Econo-Pac 10DG desalting column before treating the mixture with Cu²⁺ K₃Fe(CN)₆ at 20 °C for 1.5 h to catalyze the Cys oxidation. The presence of dimer was determined via nonreducing SDS–PAGE performed using 12.5% acrylamide gels stained with Coomassie Blue G-250.

Myosin subfragment 1 (S1) was prepared by chymotryptic digestion of rabbit myosin as described in ref 23.

Rabbit skeletal actin was purified by the method described in ref 24. Its molar concentration was determined from its absorbance at 280 nm using an $E^{1\%}$ of 1.104 cm⁻¹ and a molecular mass of 42000 Da. The preparation of pyrene-labeled actin (pyr-actin) was as previously described in ref 25. F-actin was stabilized with phalloidin by incubating a solution of 10 μ M pyr-actin with 10 μ M phalloidin overnight in 20 mM MOPS, 100 mM KCl, and 5 mM MgCl₂, pH 7.0 at 4 °C.

Cosedimentation and Quantitative Electrophoresis. Cosedimentation assays were performed at 20 °C by mixing 10 μ M actin with increasing concentrations of Tm, in the standard experimental buffer (20 mM MOPS, 100 mM KCl, 5 mM MgCl₂, pH 7.0), to a total volume of 100 μ L. The actin was then pelleted along with any bound Tm by ultracentrifugation at 100000 rpm for 20 min (Beckman Instruments TLA-100.1). Equivalent samples of pellet and supernatant were then separated by SDS–PAGE. Quantification of proteins was carried out using an Epson Perfection 1640SU scanner with a transparency adaptor attached to a PC. Scanned images were analyzed using the image-PC program (Scion Corp., Frederick, MD).

Fluorescence Titrations. Fluorescence titrations were measured at 20 °C using a Perkin-Elmer Life Sciences 50B spectrofluorometer with excitation at 365 nm with a 10 nm bandwidth and measuring emission at 405 nm with a 15 nm bandwidth. A total working volume of 2 mL was used in a 10 × 10 mm cell constantly stirred using a magnetic stirrer below the light path of the instrument. Autotitrations were made by the continuous addition of a 5 μ M S1 stock solution at a rate of 12 μ L/min using a Harvard Apparatus syringe

infusion pump 22, driving a 100 L glass syringe (Hamilton). Data were acquired over a period of 250 s, with data points being collected every 0.5 s, using an integration time of 0.45 s. Buffer solutions for the titrations were as previously described and were filtered using a 0.22 μM disposable syringe filter to remove dust particles that can produce significant noise in the stirred cell at the low levels of sample fluorescence used. Cooperative binding curves from the titrations were fitted using a two-state version of the McKillop and Geeves model (eq 1) with a varying cooperative unit size (26, 27):

$$\theta = \frac{K_1[M]K_T((1 + K_2)P^{n-1} + Q^{n-1})}{K_T P^n + Q^n} \quad (1)$$

$$\theta = (F_0 - F)/(F_0 - F_\infty) \quad (2)$$

where θ represents the fraction of total actin sites occupied, $[M]$ is the concentration of free S1 heads, n is the cooperative unit size, $P = 1 + K_1[M](1 + K_2)$, and $Q = 1 + K_1[M]$. K_T defines the equilibrium between the closed, C-state where only weak myosin binding is possible and the open, M-state that allows rigor-like binding. The fluorescence signal (F) is related to θ through eq 2 in which F_0 is the initial fluorescence before addition of S1 and F_∞ is the fluorescence at saturation with S1.

RESULTS

Affinity of AS Tropomyosin for Actin. The affinity of AS α -Tm, α -Tm, AS β -Tm, and β -Tm for actin was measured and compared to native Tm using a cosedimentation assay. An example of an SDS-PAGE gel used for determining binding affinities is illustrated in Figure 1. The two gels show an experiment involving the β -Tm protein. The top gel shows the supernatants and the bottom gel the pellets from the cosedimentation. Each sample contains 10 μM actin incubated with increasing concentrations of Tm from 0.5 μM (lane 1) to 7 μM (lane 8). In both gels actin is the upper band, and the density of this band remains practically constant in all the samples, with the majority of the actin appearing in the pellet as expected (lanes 1–8). The lower band on both gels is Tm, and the optical density of the Tm band increases from the lowest Tm concentration sample (lane 1) to the highest Tm concentration sample (lane 8). The fraction of Tm bound to actin was estimated from the density of the bands in Figure 1A and plotted in Figure 1B against the free Tm concentration. The ratio of the optical densities of the two bands in the pellet samples was 0.4 for a fully saturated actin filament, which corresponds to a binding stoichiometry of 7 actins for each Tm bound. The $K_{50\%}$ values were determined after fitting the curves with the Hill equation (eq 3), and the values are given in Table 1:

$$\theta = [\text{Tm}]^h / (K_{50\%}^h + [\text{Tm}]^h) \quad (3)$$

where h is the Hill coefficient, θ is the fractional saturation of actin with Tm, and $K_{50\%}$ is the Tm concentration required for 50% saturation of actin.

The Tm's containing the N-terminal AS extension, which is added to mimic acetylation, bind with much greater affinity

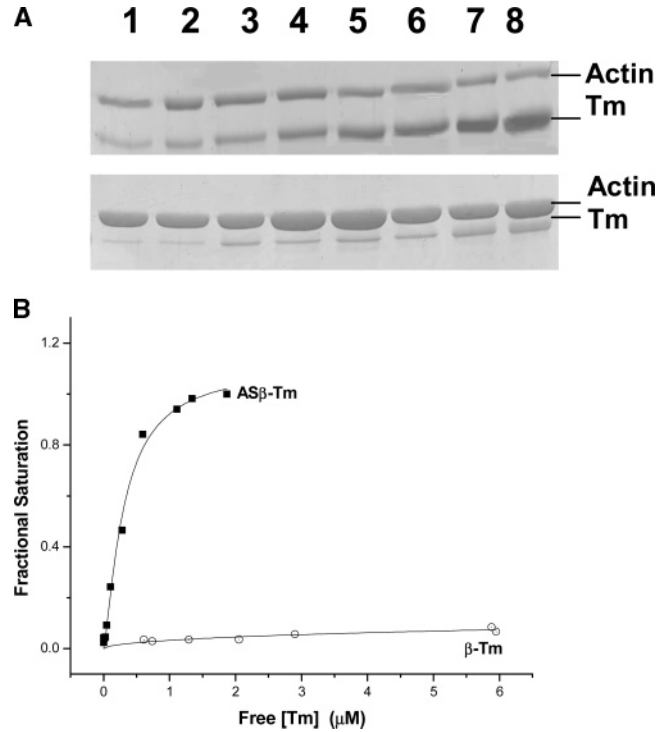


FIGURE 1: Sedimentation assay of tropomyosin binding to actin. (A) 10 μM actin and different β -Tm concentrations (0.5–7.0 μM) were spun at 100000g and the supernatants (upper gel) and pellets (lower gel) analyzed by SDS-PAGE. (B) Analysis of the fraction of β -Tm binding to actin from the relative band densities. Binding constants ($K_{50\%}$), the fraction of Tm bound to actin, were estimated from the density of the bands in the pellet and plotted against the free Tm concentration. The fitted line is the least-squares best fit to the Hill equation. Conditions: 100 mM KCl, 5 mM MgCl_2 , 20 mM MOPS, pH 7.0, 20 $^\circ\text{C}$.

Table 1: Dissociation Constants for the α - and β -Tm Constructs with and without the AS Extension^a

Tm	$K_{50\%}$ (μM)	h	K_T	n
AS α -Tm	0.035	2.65	0.1–0.25	5–9
α -Tm	2.22	1.42	0.12	5–9
AS β -Tm	0.30	1.33	0.05–0.15	5–9
β -Tm	>20		ND	ND
AS α /AS β -Tm	0.03	3.05	0.1–0.25	5–9
native gizzard Tm	0.04	1.37	0.1–0.3	5–9
sk AS α -Tm	0.18 ^b	2.05	0.1 ^c	7 ^c
sk AS β -Tm	0.23 ^b	1.6	ND ^d	ND

^a $K_{50\%}$ values and the Hill coefficient (h) were calculated via the Hill equation from the Tm/actin binding plot featured in Figure 1 and were measured from the best fit to the Hill equation as the free Tm concentration at which the actin filament is half-saturated by Tm. The errors on the fits to the data were all less than 10% of the value for $K_{50\%}$ but much larger for h : 15–30% for values of 1–1.5 and 30–50% for values above 1.5. Thus differences in the values of h are not significant. K_T and n values were obtained from the fit of the sigmoid titration curves to the two-state model of McKillop and Geeves. ^b Data from Boussouf et al. (submitted for publication). The affinities without an AS extension were >20 μM . ^c Data from Maytum et al. (28). [KCl] = 200 mM. ^d ND, not determined.

(>100-fold) than the equivalent proteins containing no AS extension. AS α -Tm binds to actin with an affinity ($K_{50\%}$ = 35 nM) which is similar to native Tm ($K_{50\%}$ = 40 nM), thus suggesting that the N-terminal AS extension is a good mimic for N-terminal acetylation in the smTm system. AS β -Tm had an affinity ($K_{50\%}$ = 300 nM) which is 10 times weaker than that of the AS α -Tm. In contrast, β -Tm did not show any

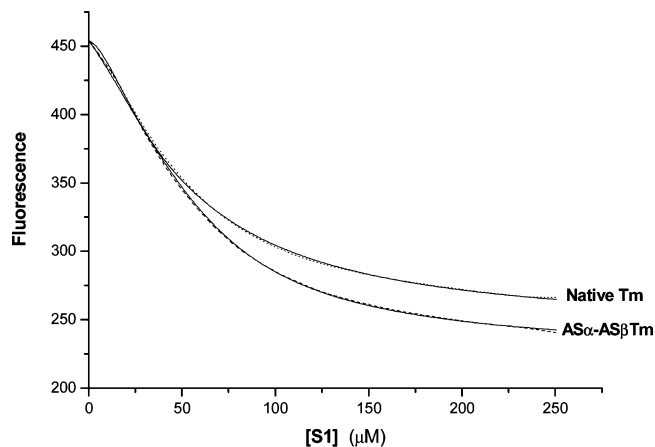


FIGURE 2: Fluorescence titration of pyrene actin by S1 in 50 nM pyrene actin plus 2 μ M native (dots) and AS α -AS β Tm (dashes) titrated with S1 between 0 and 250 nM. The data were fitted to the two-state cooperative binding model with the fits superimposed as a solid line on the raw data. Fitted parameters are given in Table 1. Conditions: 100 mM KCl, 5 mM MgCl₂, 20 mM MOPS, pH 7.0, 20 °C.

significant binding to actin; therefore, $K_{50\%}$ is greater than 20 μ M, again demonstrating the importance of the N-terminal acetylation and the equivalence of the AS extension.

Regulation of S1 Binding to Pyr-Actin by AS-Tm. Titration of the fluorescent pyrene-labeled actin with S1 was used under equilibrium binding conditions to assess whether the expressed Tm's regulate the binding of S1 to actin. Figure 2 shows the raw titration curves in which 5 μ M S1 is continuously titrated into a cuvette containing 50 nM phalloidin-stabilized pyrene actin, saturated with Tm (2 μ M added). The sigmoidal shapes of the curves show that Tm regulates S1 binding to actin. These curves were fitted to a two-state version of the three-state model. The value of K_2 (the rigor isomerization) has previously been determined to be 200; K_1 (the weak binding) was seen to be 1.8×10^5 s⁻¹ M⁻¹ for all of the reconstituted filaments. The shape of the curves is therefore defined by the value of K_T ($[M]/[C]$ equilibrium) and n (the apparent cooperative unit size). Note that the values of K_T and n are not independent; equivalent fits indistinguishable from the data can be obtained with a range of n and K_T values, which are indicated in Table 1. Larger values of n require smaller values of K_T . Table 1 shows the K_T values that were measured for each Tm after fitting to the two-state model. The range of values given in Table 1 for native Tm are in the same range as those previously reported (28). However, at the lower KCl concentration used here to ensure binding of the non-AS Tm to actin, the resolution of the parameters in the fit is not as precise as previously reported.

The K_T values for the Tm's containing the AS extension were similar to that of native Tm, therefore reinforcing the cosedimentation data in suggesting that the AS extension is a good mimic for N-terminal acetylation.

Production of Heterodimers. To verify the assembly of heterodimers from the mixture of $\alpha\alpha$ and $\beta\beta$ homodimers, using the conditions described in Experimental Procedures, SDS-PAGE was used (Figure 3). The presence of $\alpha\beta$ heterodimer is confirmed by its inability to be cross-linked. Lanes 1 and 4 contain the $\alpha\alpha$ and $\beta\beta$ smooth homodimer species which show the presence of cross-linked species

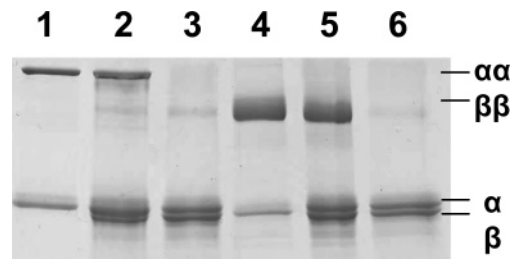


FIGURE 3: Verification of formation of $\alpha\beta$ heterodimers by gel electrophoresis after cross-linking treatment. SDS-PAGE of cross-linked Tm dimers were run under oxidizing conditions. Lanes: 1, $\alpha\alpha$ homodimer alone; 2, a 1:1 mixture of $\alpha\alpha$ homodimer with $\alpha\beta$ heterodimer; 3, $\alpha\beta$ heterodimer; 4, $\beta\beta$ homodimer; 5, a 1:1 mixture of $\beta\beta$ homodimer with $\alpha\beta$ heterodimer; 6, $\alpha\beta$ heterodimer. All samples were denatured in 20 mM DTT at 60 °C and left to cool to 37 °C before cross-linking at 20 °C via a Cu²⁺-catalyzed K₃-Fe(CN)₆ reaction.

(upper band) as well as some un-cross-linked species (lower band). The cross-linking of the homodimer species occurs between the two cysteine residues at position 190 in $\alpha\alpha$ and position 36 for $\beta\beta$. The formation of $\alpha\beta$ from the treated $\alpha\alpha/\beta\beta$ mixture is shown in lanes 3 and 6 (labeled $\alpha\beta$) by the lack of a high molecular mass band in the upper region of the gel. The heterodimer cannot form disulfide bonds because the single cysteines on each chain are too far apart (at positions 36 and 190). Instead, the presence of two bands with slightly different mobility is seen, which correspond to α and β monomer chains. Lanes 2 and 5 show the result of treatment of the mixtures of $\alpha\alpha + \alpha\beta$ and $\beta\beta + \alpha\beta$. The data indicate that only homodimers can be cross-linked. Thus, a relatively pure sample of $\alpha\beta$ smooth tropomyosin heterodimer can be prepared using the protocols described in Experimental Procedures. The presence of a small amount of $\beta\beta$ smooth homodimer species present within the $\alpha\beta$ smooth heterodimer sample is due to a slight excess of $\beta\beta$ smooth homodimer added before chain exchange. As can be seen below, the $\beta\beta$ smooth homodimer species binds actin with an affinity \sim 100-fold weaker than the $\alpha\beta$ smooth heterodimer, so that the occurrence of a small amount of $\beta\beta$ smooth homodimer will not significantly affect the binding of the $\alpha\beta$ smooth heterodimer species to actin.

Heterodimers expressing different combinations of N-terminal AS extension were also made using the method described above. $\alpha\beta$ heterodimers expressing an N-terminal AS extension on the α chain but not the β chain, termed AS α -Tm/ β -Tm, and an $\alpha\beta$ heterodimer expressing an AS extension on the β chain but not the α chain (α -Tm/AS β -Tm) were made. These heterodimers were then used in cosedimentation assays as described above to measure their actin binding affinity.

Affinity of Actin for Tm Heterodimers. The binding of $\alpha\beta$ -Tm containing different combinations of N-terminal AS extensions to actin was compared to that of native Tm using the cosedimentation assay previously described. Figure 4 plots the cooperative binding curves of AS α -Tm/AS β -Tm compared to native Tm. These curves were fitted with the Hill equation, and the concentration of free Tm at which half the actin becomes saturated ($K_{50\%}$) was determined. The $K_{50\%}$ values obtained (Table 2) demonstrate that AS α -Tm/AS β -Tm binds actin with an affinity which is similar to native Tm. Table 2 also reports the $K_{50\%}$ values obtained for $\alpha\beta$ -Tm's containing different combinations of N-terminal AS

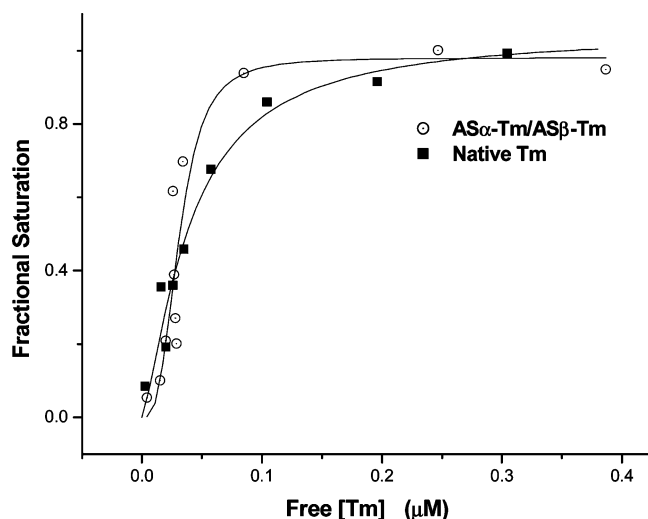


FIGURE 4: Affinities of AS α -Tm/AS β -Tm (open circles) compared with native gizzard Tm (squares) for actin as determined by cosedimentation analysis. Conditions are the same as described in Figure 1.

Table 2: Dissociation Constants ($K_{50\%}$) for the AS α -Tm/AS β -Tm Heterodimer Compared to Heterodimers with a Single AS Extension

Tm	$K_{50\%}$ (μ M)
AS α -Tm/AS β -Tm	0.03
AS α -Tm/ β -Tm	0.54
α -Tm/AS β -Tm	1.21
native gizzard Tm	0.04

extensions compared to native Tm. The $K_{50\%}$ values which were obtained were 540 nM for AS α -Tm/ β Tm and 1.21 μ M for α -Tm/AS β -Tm, respectively. These results suggest that, in order for $\alpha\beta$ -Tm to have native-like properties, it requires an N-terminal AS extension on both α and β chains. The reduction in the affinity for actin of α -Tm/AS β -Tm compared to AS α -Tm/ β -Tm suggests that the α chain dominates actin binding; however, an N-terminal AS extension on both α and β chains is required for native-like actin binding.

DISCUSSION

We have expressed α - and β -smTm with and without the AS N-terminal extension that has been used as a mimic of the N-terminal acetylation of the native Tm. Acetylation is a requirement for effective binding of Tm to actin (11, 19, 20). The extent of the affinity change has not been previously documented. Previous studies have used skTm, and there was no detectable binding for the Tm with an unmodified N-terminus (20). SmTm has a higher affinity for actin than skTm (29), and we show here differences of affinity of α -Tm to actin for native and wild type with and without the AS extension. In the case of the $\alpha\alpha$ homodimers, the AS extension increased the affinity by 100-fold and gave an affinity within a factor of 2 of the affinity of the native $\alpha\beta$ -Tm. A similar increase in affinity was seen for the $\beta\beta$ homodimer although the unmodified dimer had an affinity that was too weak to define with any precision. The 100-fold increase in affinity for the AS extension is consistent with an initiation polymerization model of Tm binding to actin (30) where the end-to end interactions play a major role in defining the polymerization of Tm on actin.

Note that the data in Table 1 indicate that the actin affinity of the AS β -Tm is very similar for both skeletal and smooth

muscle isoforms. This is not true for α -isoforms where the smooth muscle AS α -Tm has an actin affinity five times that of the skeletal isoform. Native smooth muscle Tm ($\alpha\beta$) also has a tighter affinity for actin than native skeletal β -Tm, confirming that the actin affinity is dominated by the α -isoforms in the $\alpha\beta$ heterodimers. α - and β -Tm isoforms are each derived from a single gene via alternate splicing (2, 3). The α -isoforms differ at exons 2 and 9, while the β -isoforms differ at exons 6 and 9 (2, 3). The tighter actin affinity and stronger regulation of smTm vs skTm may result from the exon 9 differences as this appears to affect the Tm–Tm interactions.

We also demonstrate that the AS-smTm can be successfully assembled into $\alpha\beta$ heterodimers as has previously been shown for the native protein (8, 9). The $\alpha\alpha$ homodimer and the $\alpha\beta$ heterodimer carrying the AS extension have similar affinities to actin ($K_{50\%} = 0.03$ M), which are also similar to the native $\alpha\beta$ -Tm ($K_{50\%} = 0.04$). Using this approach, we have assembled heterodimers in which only one of the two chains carries the AS extension; AS α -Tm/ β -Tm and α -Tm/AS β -Tm. The absence of the AS on the β -Tm results in an ~ 8 -fold weaker affinity of the heterodimer for actin, while the absence of AS on the α -Tm gave an ~ 40 -fold weaker affinity, only 2-fold tighter than that for the $\alpha\alpha$ non-AS homodimer. Thus the α -Tm and the AS on the α -Tm dominate the actin binding properties of the heterodimer. Clearly both AS extensions are required to produce native-like actin affinities, but a single AS is sufficient to have a major effect on actin binding. This is consistent with the view that loss of actin binding without acetylation/AS extension is due to charge repulsion between the two +ve charges on the N-terminal amine groups (13). Removal of one charge may be sufficient to eliminate most of the repulsion to allow a native-like structure of the N-terminus and Tm–Tm overlap.

The data on the titration with S1 show that once the filament is assembled, all of the Tm's induce sigmoid binding curves for S1, consistent with Tm occupying one of two states which sterically blocks the site on the thin filament where S1 binds (27, 31). In the McKillop and Geeves model this is called the C-state (closed or calcium-induced state), and K_T defines the equilibrium between the C-state and the open or myosin-induced state. The values of K_T and n are of the same order for all of the smTm's; $\alpha\alpha$, $\beta\beta$, and $\alpha\beta$ are all similar, and the value of K_T is not greatly affected by the presence of the AS extension. All of the Tm's are therefore predicted to occupy the same C-state (calcium-induced or closed state) of the actin filament as seen for native skTm and smTm. Thus, although the AS extension and acetylation have a marked effect on the affinity of the Tm for actin, once assembled the behavior of Tm on the actin surface is not greatly influenced by the N-terminal modification.

There are significant differences in the way in which skTm and smTm influence S1 binding to actin, and analysis of titration curves suggests that this is primarily due to greater cooperativity in the filaments (larger n) (29). We have interpreted this as due to stronger Tm–Tm contacts which allow greater communication between actin–Tm structural units (29). The stronger Tm–Tm contacts in smTm also give rise to the tighter affinity of smTm for actin and in the higher viscosity of smTm vs skTm (29). This increase in viscosity

is also seen in the difference between AS and unacetylated Tm (20) and supports stronger end-to-end interactions as the major effect of acetylation/AS extension. This greater cooperativity in smTm is also reflected in actomyosin ATPase and motility data (29). The data presented here demonstrate that the N-terminal modification has a marked affect on binding but little effect on the cooperativity and regulation of S1 binding. This suggests that the increased cooperativity in smTm is not simply a result of stronger Tm–Tm contacts.

This study was only possible because of the high affinity of α - and $\alpha\beta$ -smTm for actin. Such a study cannot be made for skTm without an N-terminal modification because like $\beta\beta$ -smTm there is no significant binding of skTm ($\alpha\alpha$, $\beta\beta$, or $\alpha\beta$) to actin ($K_{50\%} > 20 \mu\text{M}$).

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