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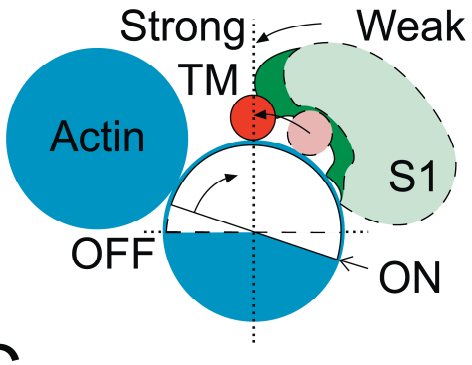
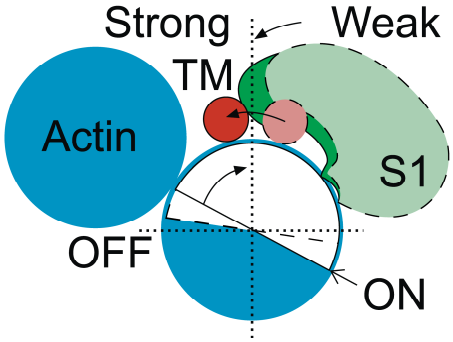
A

Actin-WT TM-S1



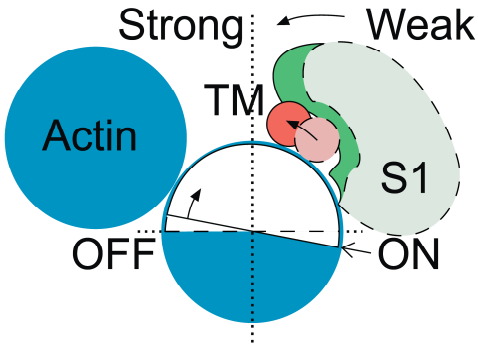
B

Actin-K168E TM-S1



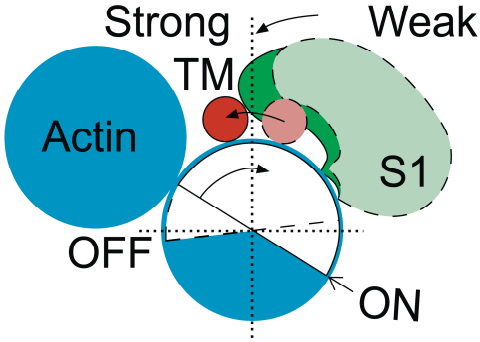
C

Actin-R167H TM-S1



D

Actin-R167G TM-S1



Abnormal movement of tropomyosin and response of myosin heads and actin during the ATPase cycle caused by the Arg167His, Arg167Gly and Lys168Glu mutations in TPM1 gene

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Short title: Effects of mutations in Tpm1.1

Keywords: tropomyosin; F-actin; myosin; troponin; ghost muscle fibres; congenital myopathies; fluorescence polarization.

Abbreviations used: TM, tropomyosin; WTTM, wild-type tropomyosin; S1, myosin subfragment 1; FITC-phalloidin, phalloidin-fluorescein isothiocyanate; 1,5-IAEDANS, N-(iodoacetaminoethyl)-1-naphthyl-amine-5-sulfonic acid; 5-IAF, 5-iodoacetamidofluorescein; DTT, dithiothreitol; AMP-PNP, adenosine 5'-(β,γ-imido)triphosphate tetralithium salt hydrate.

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ABSTRACT

Amino acid substitutions: Arg167His, Arg167Gly and Lys168Glu, located in a consensus actin-binding site of the striated muscle tropomyosin Tpm1.1 (TM), were used to investigate mechanisms of the thin filament regulation. The azimuthal movement of TM strands on the actin filament and the responses of the myosin heads and actin subunits during the ATPase cycle were studied using fluorescence polarization of muscle fibres. The recombinant wild-type and mutant TMs labelled with 5-IAF, 1,5-IAEDANS-labelled S1and FITC-phalloidin F-actin were incorporated into the ghost muscle fibres to acquire information on the orientation of the probes relative to the fibre axis. The substitutions Arg167Gly and Lys168Glu shifted TM strands into the actin filament centre, whereas Arg167His moved TM towards the periphery of the filament. In the presence of Arg167Gly-TM and Lys168Glu-TM the fraction of actin monomers that were switched on and the number of the myosin heads strongly bound to F-actin were abnormally high even under conditions close to relaxation. In contrast, Arg167His-TM decreased the fraction of switched on actin and reduced the formation of strongly bound myosin heads throughout the ATPase cycle. We concluded that the altered TM-actin contacts destabilized the thin filament and affected the actin-myosin interactions.

Introduction

Tropomyosin (TM) is an α-helical coiled coil protein, which polymerizes end-to-end along each chain of the actin filament (F-actin) and regulates actin interactions with myosin [1]. TM sequence is divided into semi equivalent periodic repeats about 40 amino acids long, which extend along consecutive actin monomers. The N-terminal halves of each repeat contain two groups of charged amino acids, which make direct electrostatic contacts with oppositely charged residues exposed on actin [2,3].

It is well established that efficient regulation of the actin-myosin interactions requires changes in TM azimuthal position on F-actin. When bound to actin alone TM is in equilibrium between closed and blocked positions, where it partially occludes myosin-binding sites on actin [4]. Initial attachment of the myosin heads to actin filament shifts TM further to the inner domain of actin (open position) and exposes the myosin binding sites on actin [5]. Isomerization of myosin heads into a rigor state allows for stabilization of TM in the open position, which involves specific interactions between TM and the tip of myosin head [6]. It appears that the exposure of the myosin-binding sites on actin occurs not only due to the movement of TM, but also due to the rotation of actin in the opposite direction, which allows an easier access for the myosin heads to their actin-binding sites on actin. It has been found [7-10] that when TM strands move from the periphery of actin filament to its centre (from the closed/blocked to the open position), actin monomers turn to meet TM. Thus, the movement of TM strands from the closed to the open position correlates with an increase in the proportion of switched on actin monomers and an enhanced ability of actin filament to stimulate the ATPase activity of myosin. Conversely, along with the shift of TM towards the outer edge of the filament (from the open to the blocked position) the proportion of the switched on actin

monomers decreases and the ability of F-actin to stimulate the ATPase activity is reduced [7-10].

Structural elements of TM that are employed in the integration of the conformational changes associated with regulation of actin-myosin interactions are not fully understood. Mutations in specific regions of TM were found in patients suffering from various types of congenital skeletal muscle myopathies and cardiomyopathies [11,12].

In humans most of the disease-causing mutations were found in a specific tropomyosin gene. For example, in nemaline myopathy and congenital fiber type disproportion point mutations segregate with TPM3 gene, which encodes for Tpm3.12 isoform expressed in type 1 slow muscle fibers [13]. However, when introduced into TPM1 gene the mutations impair *in vitro* functions of fast skeletal Tpm1.1 in a way that is consistent with the effects observed in myopathy patients [14,15]. This suggests that the regions affected by mutations share similar functions in both TM isoforms. In the previous work we studied functional effects of Arg167His, Arg167Gly and Lys168Glu replacements in Tpm1.1 [14,15]. Arg167 is one of the positively charged residues, which form actin-binding site through electrostatic interactions with the negatively charged Asp25 exposed on the actin surface [16,17]. Although Lys168 does not interact directly with actin, is was postulated to maintain the proper position of Arg167 by electrostatic repulsion [18]. We have found that all three substitutions decreased the affinity of tropomyosin for actin, and suppressed the activation of the actomyosin ATPase activity and *in vitro* motility of actin filaments [14,15]. We hypothesized that the reduced capability of the thin filament to fully activate myosin cross-bridges was due to impaired TM interactions with actin causing an abnormal azimuthal movement of TM strands. To confirm this hypothesis and gain more insight into the phenomena associated with alteration on the thin filament caused by mutations in TM, we used the

polarized fluorescence of muscle fibres reconstituted with recombinant TMs carrying the substitutions mentioned above. We have found that the mutations indeed disturbed the azimuthal movement of TM on the filament, but the direction of the movement of the three types of TM was not uniform. The presence of the mutant TMs had different effects on actin monomers ability to rotate in the filament, a process necessary to switch the monomers on. In addition, TM mutants affected the fraction of myosin heads strongly bound to actin. The results revealed that there is no simple mechanism which governs the TM-dependent regulation of actin-myosin interactions. Depending on the location and the type of amino acid substitution, the reduced activation of contraction is caused either by blocking the access of myosin heads to actin or by increasing the fraction of strongly bound myosin heads which cannot freely enter the cross-bridge cycle.

2. Materials and methods

2.1. Preparation of proteins

Myosin was separated from fast skeletal muscles of rabbits as described by Margossian and Lowey [19]. Myosin subfragment-1 (S1) was prepared by treatment of fast skeletal muscle myosin with α-chymotrypsin for 10 min at 25^{o}C according to Okamoto and Sekine [20]. Modification of the reactive residue Cys707 of S1 with 1,5-IAEDANS (Molecular Probes, actin-AEDANS) was carried out as described previously [21]. The degree of Cys707 modification was 0.90–0.95.

Recombinant wild type and mutant Tpm1.1 carrying Arg167His, Arg167Gly or Lys168Glu substitutions were expressed in BL21 (DE3) cells and purified as described before [22]. All tropomyosins had an extension of two additional amino acids (AlaSer), which compensated for the reduced affinity of recombinant non-acetylated skeletal TM

to F-actin. TM labelling with 5-IAF at Cys190 was performed as described previously [10,23], producing a probe to protein molar ratio 0.8:1.

2.2. Preparation and labelling of ghost fibres

Experiments were performed at the animal care facility of the Institute of Cytology RAS. Adult male New Zealand white rabbits (3–4 kg) were killed by sodium pentobarbitone injection (200 mg/kg) in accordance with the official regulations of the community council on the use of laboratory animals, and the study was approved by the ethics committee for animal experiments. The psoas muscle was exposed ventrally and a bundle of about 100 fibres was gently separated from the muscle. The fibers were glycerinated using the method of Rome [24]. Ghost fibres were prepared by incubation of single glycerinated skeletal fibres for 1.5 h in 800 mM KCl, 1 mM $MgCl_2$, 10 mM ATP, 67 mM phosphate buffer, pH 7.0 [8]. Actin accounted for 80% of the overall protein content of the ghost fibres. S1 and TM were incorporated into pure actin filaments by incubation of the fibre in a standard solution containing 50 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 10 mM Tris-HCl, pH 6.8, and the respective protein in concentration 1.0-2.5 mg/ml. The unbound proteins were washed out by incubation of the fibres in the same buffer without proteins. FITC-phalloidin was dissolved in methanol, pre-aliquoted and then methanol was evaporated. FITC-phalloidin was conjugated with F-actin of the fibres by their incubation in a standard solution containing 40 μM FITC-phalloidin for 2.5 h at room temperature [8,10].

The effectiveness of reconstitution of the filaments in ghost muscle fibres used for fluorescent measurements was verified by SDS-PAGE [25]. The fibres in the final preparations contained actin, S1, recombinant TM and Z-line proteins. The molar ratio of WTTM or mutant TMs to actin was 1:6.5 (±2) irrespective of whether TMs were

modified by 5-IAF or not. In the absence of nucleotides and in the presence of MgADP, MgAMP-PNP, and MgATP the molar ratios of S1 to actin were 1:5 (±2), 1:5 (±2), 1:8 (±2), and 1:14 (±2), respectively.

2.3. Fluorescence polarization measurement

Steady-state fluorescence polarization measurements on single ghost muscle fibres were made using a flow-through chamber and polarized microfluorimeter [26]. The polarized fluorescence from 1,5-IAEDANS-labelled S1 was excited at 407±5 nm, and from FITC-phalloidin-labelled actin and 5-IAF-labelled TM, at 437±5 nm and recorded at 500-600 nm. The intensities of four components of polarized fluorescence $_{\parallel}I_{\parallel}$, $_{\parallel}I_{\perp}$, $_{\perp}I_{\perp}$ and $_{\perp}I_{\parallel}$ were detected by two photomultiplier tubes. The subscripts ‖ and ⊥ designate the direction of polarization parallel and perpendicular to the fibre axis, the former denoting the direction of polarization of the incident light and the latter that of the emitted light (Fig. 1A). Fluorescence polarization ratios were defined as: $P_{\parallel} = (_{\parallel}I_{\parallel} - _{\parallel}I_{\perp})/(_{\parallel}I_{\parallel} + _{\parallel}I_{\perp})$ and $P_{\perp} = (_{\perp}I_{\perp} - _{\perp}I_{\parallel})/(_{\perp}I_{\perp} + _{\perp}I_{\parallel})$. Measurements were carried out in a standard solution containing 50 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 10 mM Tris-HCl, pH 6.8 in the absence or presence of 1.0 mM ADP, 16 mM AMP-PNP or 5 mM ATP. 10 mM creatine phosphate and 140 unit/ml creatine kinase were added to the solution containing ATP. The concentration of $MgCl_2$ was 3 mM when experimental medium contained ADP or no nucleotides, 8 mM in the presence of ATP, and 18 mM in the presence of AMP-PNP. The absence of nucleotides or the presence of MgADP mimicked strong binding of myosin heads to F-actin (AM and AM^·ADP states), and the presence of MgAMP-PNP or MgATP mimicked weak myosin binding (AM'·ADP and AM*·ATP states) [27]. We used solutions with lower ionic strength than those in

situ, in order to increase the affinity of myosin S1 to actin at simulated weak-binding states. It is known that the degree of regulation can be decreased under low ionic strength conditions. To avoid the decrease in the degree of regulation it has been suggested that solutions containing 2-5 mM Mg^{2+} are used [28]. In our experiments all solutions contained 2-3 mM free Mg^{2+}.

To assess quantitative changes in the probe orientation, we used the "helix plus isotropic model" [21,26,29,30]. In this model, it is assumed that there are two populations of probes in a fiber – the population of randomly distributed fluorophores (present in an amount of N) and the population of the ordered fluorophores (present in an amount of 1–N). The axes of dipoles of the randomly distributed probes are oriented at the magic angle of 54.7° relative to the long axis of the actin filament, whereas the axes of dipoles of the ordered probes are at angles different than 54.7° and are arranged in a spiral along the surface of the cone, the axis of which coincides with the long axis of the actin filament. The dipoles of fluorescence absorption and emission form the angles Φ_A and Φ_E, respectively (Fig.1B), at the top of the cone. Motions of probe relative to protein are included in the model as the angle γ (the angle between the absorption and emission oscillators, Fig. 1B). The value of γ is constant for the probe and is assumed to be 17° for 5-IAF bound to tropomyosin, 14° for FITC-phalloidin bound to F-actin, and 20° for 1,5-IAEDANS bound to S1 [26]. The actin filament is assumed to be flexible. The maximal angle of its deviation from the fibre axis is designated as $\theta_{1/2}$ (Fig. 1A)[30].

Since a probe upon its attachment to a protein molecule can become available for a solvent as well as affected by adjacent amino acid residues, the orientation and mobility of absorption and emission dipoles of the probe may be also sensitive to a change in its local environment. The information about such changes can be obtained by analyzing the

fluorescent spectrum of the probe. In our work, we measured the position of the maximum of the fluorescence spectrum in all the experiments with an accuracy of 0.3 nm, and did not find any reliable shifts of the spectrum of the proteins modified by 5-IAF, 1,5-IAEDANS and FITC-phalloidin. Based on these data was the suggestion that the changes in polarized fluorescence registered in our experiments reflected mainly the changes in orientation and mobility of the absorption and emission dipoles of the probes.

In all our experiments the pattern of Φ_E changes was similar to that of Φ_A, therefore only Φ_E, N, and $\theta_{1/2}$ values were presented in the figures. The statistical reliability of the changes was evaluated using Student's t-test.

3. Results

3.1 Conformational states of TM, actin and myosin S1 in reconstituted muscle fibres measured by the polarized fluorescence microscopy

In line with our earlier findings [8,10], the incorporation of FITC-phalloidin-labelled actin (FITC-actin) or 5-IAF-labelled recombinant Tpm1.1 (AF-TM) into the actin filaments as well as the binding of 1,5-IAEDANS-labelled S1 (AEDANS-S1) to F-actin initiated polarized fluorescence in the ghost muscle fibres (Tables 1-3). When the helix plus isotropic model (see Materials and methods) was fitted to the fluorescence polarization data for FITC-actin, AF-WTTM, and AEDANS-S1, the values of the angle between the fibre axis and the emission dipoles of the probes (Φ_E) were found to be equal to 47.5°, 55.3°, and 44.4°, respectively (Figs. 2a,3a,5a). The relative amount of randomly oriented probes (N) was zero for AF-WTTM and did not exceed 0.20 for FITC-actin and AEDANS-S1, showing a rigid binding of the probes to their target proteins and a highly-ordered arrangement of F-actin, S1 and TM in the fibres. Similar values of Φ_E and N were obtained earlier [8,10,31].

Our data demonstrated that the flexibility of F-actin in the F-actin-TM complex was higher than that of the WTTM. The values of $\theta_{1/2}$ for F-actin and WTTM were close to 6.9° and 5.0°, respectively (Figs. 2b,3b). Flexibility values of the same order of magnitude were observed earlier [4,32]. Binding of WTTM to F-actin decreased the flexibility of the latter (the values of $\theta_{1/2}$ for F-actin decreased from 7.8° to 6.9°, $P<0.05$) (Fig. 2b). This observation is in agreement with the available data on an increase in actin's persistence length induced by TM [33,34]. The binding of the unlabelled WTTM to F-actin increased the Φ_E value for FITC-actin from 47.5° to 48.2° ($P<0.05$). As FITC-phalloidin is located in the groove of actin filament and is specifically bound to three adjacent actin subunits [35], the changes in the Φ_E value presumably reflect the changes in the helical structure of F-actin (for example, variations in the pitch of the generic and long pitch helices [36]). The increase in the Φ_E value indicating that the emission oscillators have moved away from the filament axis could be interpreted as a result of a clockwise rotation of actin monomers [8-10,31]. According to our earlier assumption [7,8,10], there are two different states of actin monomers in F-actin filaments of the ghost muscle fibres: the so-called “ON” and “OFF” states, that differ in their conformation and the capability to stimulate myosin ATPase activity. Monomers of actin in the “ON” state can activate myosin ATPase, whereas in the “OFF” state cannot [1,37]. It was suggested that for actin monomers in the “OFF” state the emission oscillators of FITC-phalloidin were turned to the filament axis as compared to their orientation typical for the “ON” state of actin monomers [7-10,31]. The clockwise rotation of actin monomers around the filament axis releases the myosin-binding sites from TM thus allowing the myosin heads to bind strongly to F-actin. On the contrary, the counter clockwise rotation of actin monomers hides these sites under TM.

3.2. The effect of the mutations in Tpm1.1 on the position of TM and the conformational state of actin in the absence of S1

To study the effects of the point mutations in Tpm1.1 on the orientation and mobility of tropomyosin and actin monomers, the actin filaments reconstituted in the ghost fibres were saturated with the wild type and mutant TMs.

Binding of the WTTM increased the Φ_E value of FITC-actin from 47.5° to 48.2° ($P<0.05$) (Fig. 2a), therefore one can suppose that the binding of the WTTM increased the fraction of the switched on actin filaments subunits [8,10]. An increase in this fraction was also observed in the presence of the unlabelled Arg167His, Arg167Gly and Lys168Glu mutant TMs, because the values of Φ_E for FITC-actin increased from 47.5° to 48.0°, 47.9 and 47.8° ($P<0.05$), respectively. However, the fraction of the switched on monomers in the presence of all the mutant TMs was smaller than in the presence of the WTTM.

For the AF-WTTM, the value of Φ_E was close to 55.3° (Fig. 3a). The binding of TM to F-actin induced a rotation of actin monomers (Fig. 2a), therefore to detect the position of TM relative to the inner or outer domains of actin one must take into account changes in spatial arrangement of the F-actin helix [10,31,36]. As the binding of WTTM to F-actin caused an increase in the Φ_E value for F-actin by 0.7° ($p<0.05$), the value of the Φ_E angle for AF-WTTM relative to the F-actin helix (corrected Φ_E) was 54.5° (Fig. 4). Similarly, the corrected values of Φ_E for the Arg167His, Arg167Gly and Lys168Glu mutant TMs were 55.0°, 53.6° and 54.3° ($p<0.05$), respectively (Fig. 4). This means that the substitutions in TM differently affected rotation of the emission dipole of fluorescent probe AF relative to the position found in WTTM. The fluorophore attached to

Arg167His-TM was rotated to the periphery of the filament. In contrast, the probe bound to the two other TM mutants was either slightly (Lys168Glu-TM) or strongly (Arg167Gly-TM) turned to the centre of the filament. As the fluorescent probe was covalently bound to TM strands, the results suggested that while the Arg167His-TM was located closer to the outer domain of actin than WTTM, Arg167Gly-TM was shifted closer to the inner domain of actin. The substitution Lys168Glu also tended to shift TM towards the inner domain of actin.

Mutation-induced changes in TM orientation on the filament correlated with the previous results which showed that the substitutions Arg167His and Arg167Gly reduced actin affinity about three-fold [14]. Most probably the decreased affinity reflects destabilization of the preferable position of TM, which allows for a shift of the mutant TMs. The effect of Lys168Glu on TM's affinity for actin was milder [14], hence the binding was not strongly distorted and TM occupied the position closer to the position of WTTM.

The inhibition of myosin binding to actin is attained via a shift of TM strands towards the outer domains of actin [4]. As the Arg167His-TM was located closer to the outer domain of actin than the WTTM, the former is expected to exert a greater steric hindrance for the strong S1 binding leading to reduced actomyosin ATPase activity. Conversely, the Arg167Gly and Lys168Glu mutant TMs are presumed to be closer to the inner actin domains than the WTTM (Fig. 4), which might facilitate cross-bridge cycling and the actomyosin ATPase activity. Thus, the Arg167His mutation may inhibit the ATPase activity of acto-S1 to a larger extent than the Lys168Glu and Arg167Gly mutation. These predictions do not agree with the previous data showing that all three mutations inhibited the ATPase activity even at very high S1 concentrations [14]. Thus,

steric blocking of the myosin binding sites on actin is not the only mechanism that is disturbed by the disease-associated mutations in TM.

The data demonstrated in this work showed that the point mutations distorted the ON-OFF equilibrium of actin monomers. In the presence of mutant TMs the fraction of actin subunits that were switched off was higher than in the presence of WTTM (Fig. 2). This implies that by shifting the monomers towards OFF state all mutant TMs were able to inhibit binding of myosin heads and the cross-bridge cycle in ghost muscle fibres.

As shown in Fig. 3b, all mutations in TM decreased the values of $\theta_{1/2}$ for polarized fluorescence of AF-TM by 1.1° for Arg167His-TM, 0.9° for Lys168Glu-TM and 0.7° for Arg167Gly-TM ($p<0.05$). This means that the mutations decreased the flexibility of TM strands on the filaments. As flexible chains can be shifted into different functional positions more easily [38], this finding provides a good explanation of the inhibitory behaviour of the mutant TMs.

3.3. Spatial arrangement of the wild-type tropomyosin, actin monomers and myosin heads during the ATPase cycle

The binding of S1 to F-actin in the presence of nucleotides had a pronounced effect on the Φ_E, $\theta_{1/2}$, and N parameters of polarized fluorescence for AF-TM, FITC-actin and AEDANS-S1 (Figs. 2-5). Specifically, the N values for S1 increased from 0.182 in the absence of nucleotides to 0.549 relative units in the presence of MgATP (Fig. 5b). The increase in the value of N may result from a decrease in S1 affinity for F-actin [8,10]. Upon MgATP binding to S1 the $\theta_{1/2}$ values for FITC-actin-WTTM decreased from 6.5° to 6.3° (Fig. 2b) and for AF-WTTM from 5.0° to 4.6° ($p<0.05$) (Fig. 3b). Our data have shown that the nucleotides themselves have no marked effect on the position and flexibility of TM and F-actin in the absence of S1 (the values of Φ_E and $\theta_{1/2}$ for AF-TM,

FITC-actin do not change, data not presented), therefore the changes in the flexibility of these proteins may primarily result from the changes in their conformation, which occur upon the binding of myosin heads to F-actin and formation of electrostatic interactions between F-actin, myosin and TM suggested by the results of other investigations [6].

For FITC-actin the transition from strong myosin binding state observed in the absence of nucleotides (AM) to the weak binding state in the presence of MgATP (AM*·ATP) was accompanied by a decrease in the Φ_E value (from 50.4° to 46.8°) (Fig. 2a). During the AM to AM*·ATP transition the values of Φ_E observed for AF-WTTM increased from 54.9° to 55.3° (Fig. 3a) and for AEDANS-S1 increased from 44.4 to 47.6° (Fig. 5a). The corrected Φ_E values for AF-WTTM increased at the transformation from AM to AM*·ATP from 52.0° to 56.0° (Fig. 4). It is known that while 5-IAF and 1,5-IAEDANS are rigidly linked to the Cys190 of TM and the Cys707 of S1, FITC-phalloidin is located in the F-actin groove and specifically binds to three adjacent subunits of F-actin [35]. Thus, the changes in Φ_E for these probes can be interpreted as tilting of the myosin head or SH1 helix from the surface of the filament, a shift of TM strands to the periphery of the filament, and a turn of actin subunits towards the centre of the filament, respectively. In line with our earlier findings, a decrease in Φ_E values for FITC-actin and an increase in Φ_E values for AEDANS-S1 may be interpreted as a decrease in the fraction of the switched on actin monomers and a transition of the myosin heads to weak binding with F-actin [8,10]. Hence, during the ATPase cycle a decrease in the amount of the myosin heads strongly bound to F-actin was accompanied by a shift of TM strands towards the outer domain of actin and a decrease in the fraction of the actin monomers in the ON state. It is noteworthy that each intermediate state of the myosin heads induced a definite conformational state and a specific position of actin subunits and TM strands in the filament (Figs. 2-5). Nevertheless EM data do not show

any evidence of actin twisting or rotation [4-6]. However both EM and our data give evidence of TM movements. One of the possible reasons for the discrepancy between the data on F-actin obtained by these two methods is that we use the muscle fibers containing native proteins, while EM experiments demand the fixing of the material.

3.4. The effect of the mutations in Tpm1.1 on the position of TM strands, the conformational state of actin, and binding of S1 to F-actin during the ATPase cycle

The data illustrated in Figures 2-5 show that the three studied substitutions in the TM molecule markedly changed the position of TM on the filament and the orientation of the myosin heads and actin monomers during the ATPase cycle.

The amino acid substitutions affected the Φ_E values of AF-TM. In the presence of the nucleotides mimicking the ATP hydrolysis steps, the values of Φ_E were higher for Arg167His-TM, but much lower for the Arg167Gly-TM and Lys168Glu-TM than for WTTM (Figs. 3,4). This implies that during the ATPase cycle the mutation-dependent positions of TM were similar to the positions observed in the absence of myosin S1.

The shift of the Lys168Glu-TM and Arg167Gly-TM towards the inner domain of actin (lower Φ_E in Figs. 3a and 4) was accompanied by an increase in the fraction of the strongly bound myosin heads (lower Φ_E and N in Fig. 5) and an increase in the relative amount of actin monomers that were in the ON state (higher Φ_E in Fig. 2a). Thus, despite the fact that in the presence of MgAMP-PNP and MgATP most of the actin monomers were rather switched off (Fig. 2a, Φ_E is lower than in the absence of nucleotides), in the presence of Lys168Glu-TM and Arg167Gly-TM a certain amount of myosin heads retained the ability to bind strongly to actin, i.e. to form cross-bridges similar to those typically observed in the presence of MgADP. This was not the case with WTTM or Arg167His-TM: in the presence of MgAMP-PNP and MgATP

switching the actin monomers off correlated with the formation of weakly-bound cross-bridges.

Surprisingly, in the presence of all TM mutants the values of N for S1-AEDANS were lower throughout the ATPase cycle than in the presence of WTTM (except for Arg167His-TM in the presence of MgAMP-PNP) (Fig. 5), which indicated a more ordered conformation of myosin heads. Because myosin was shown to bind electrostatically to TM [6], the lower values of N may result from the altered interaction between myosin heads and mutant TM. Similar changes in the parameters of Φ_E and N were observed previously for the muscle fibers at rigor [39]. Thus we suggest that in the fibers with the Lys168Glu-TM and Arg167Gly-TM incorporated, some myosin heads can retain their ability to bind strongly to F-actin even in the presence of ATP.

During the ATPase cycle the values of $\theta_{1/2}$ for FITC-actin (Fig. 2b) and for AF-TM (Fig. 3b) were lower in the presence of all mutant TMs than in the presence of WTTM. As nucleotides themselves have no marked effect on the flexibility of TM and actin in the absence of S1 [8,10], the changes in the flexibility of F-actin and TM may also be due to result from the changes in the conformation of these proteins, which occur upon the binding of myosin heads to F-actin and the formation of electrostatic interaction between F-actin, myosin and TM [4,6].

4. Discussion

Mutations in TPM1 gene were used to create a model system for studying mechanisms involved in the regulation of actin-myosin interactions by tropomyosin. We selected three substitutions: Arg167His, Arg167Gly and Lys168Glu, which were shown to reduce the affinity of Tpm1.1 for actin, lower activation of actomyosin ATPase in solution, decrease velocity of the thin filaments in in vitro motility assay [14,15], and

reduce force production in muscle fibres [40]. In humans the mutations were found in Tpm3.12 expressed in slow muscle fibers and were associated with congenital myopathies, skeletal muscle diseases that are characterized by hypocontractile phenotype and muscle weakness [41]. Although muscle dysfunction observed in patients carrying the same mutations in Tpm3.12 are consistent with the results obtained *in vitro* with Tpm1.1, the two isoforms differ in 25 amino acid residues plus an extra N-terminal Met, thus direct extrapolation of the data obtained with the use of Tpm1.1 to human slow skeletal muscle fibers may not be justified.

Targeting fluorescent probes to the specific sites within TM, actin and myosin subfragment S1, which were reconstituted into muscle fibres, enabled us to study the effects of the substitutions on conformational changes within the proteins interacting during the ATP hydrolysis by the myosin heads. To investigate involvement of TM-actin interface in the regulation of actin-myosin interactions we used a simplified regulatory system lacking the Tn complex. Using polarized fluorescence we collected a set of data, which revealed a complex pattern of TM, actin and myosin heads responses to the point mutations in TM, which affect the actin-TM interface. The differences in orientation of the contractile proteins induced by the three substitutions are summarized in Fig. 7.

Each mutation specifically affected the azimuthal movement of TM on the actin filament during the ATPase cycle. As compared to the WTTM, the mutant Lys168Glu-TM and Arg167Gly-TM were more shifted towards the inner domain of actin under conditions simulating the strong and weak myosin binding to actin. This shift was accompanied by a rotation of actin monomers leading to a larger fraction of switched on actin monomers. In addition, the presence of Lys168Glu-TM and Arg167Gly-TM on the filament invoked abnormal responses of myosin heads by increasing the fraction of

strongly bound myosin heads, which did not enter the cross-bridge cycle. On the contrary, the Arg167His mutation stabilized TM strands near the outer domain of actin at all stages of the cycle. This transition shifted larger fraction of actin monomers into OFF state and reduced the number of strongly bound myosin heads.

Because the only differences between the reconstituted muscle fibers were single amino acid residues located within one actin-binding site of TM, the observed changes in the orientation of the proteins must stem from altered TM-actin interactions. TM is a two-chain α-helical coiled coil with a seven amino acid long heptad periodicity required to build the characteristic “knobs into holes” structure at the interface between the two α-helices [42]. The residues in the heptad repeat are labelled from “a” to “g”. While the “a“ and “d“ residues are hydrophobic and form the core of the molecule, the “e“ and “g“ residues are oppositely charged and stabilize the coiled coil through interchain electrostatic interactions. Binding of TM to actin involves seven periodic sites along TM coiled coil, in which conservative residues located in the “b”, “c” and “f” positions are properly oriented to interact with actin [43].

The Arg167 is in the “f” position within the fifth actin-binding period. Because the nature of interactions between TM and actin is electrostatic [4,44], the substitution of Arg167 changes the electrostatic energy landscape of TM and disrupts its contacts with negatively charged residues in actin [45], which may decrease the affinity of TM for F-actin. Indeed, weakening of the links between TM and F-actin has been observed earlier [14]. This suggests that a decrease in the affinity of TM for F-actin entails destabilization of TM position on actin.

A similar effect on the movement of TM strands and myosin heads and actin orientations during the ATPase cycle was observed earlier in polarized fluorescence experiments, in which Tpm2.1 deletion mutant (Glu139del-TM) was used [10]. Glu139

is the “f” position residue, which interacts electrostatically with a positively charged cluster on actin [16], thus the deletion leads to a local reduction in the surface charge of TM and a decrease in the affinity of Glu139del-TM for F-actin [46]. Taken together, the Arg168Gly and Glu139del mutations suggest that the reduction of charge within the actin-binding sites destabilizes the TM-actin complex and leads to a decrease in actin affinity, which shifts TM strands towards the open position [8,10]. However, comparing two different TM mutants with substitutions in Arg167 allowed us to demonstrate that destabilization of TM-actin contacts does not necessarily lead to similar responses in TM, actin and myosin S1 orientation. While replacement of Arg with Gly facilitated a displacement of TM towards the inner domain, the substitution of Arg chain with His shifted TM towards actin outer domain. Various behaviours of TM mutants could stem from differences in charge in the consensus actin-binding site. Substitution of Arg167 with Gly neutralizes the positive charge, which may facilitate the shift towards the inner domain of actin. The replacement of Arg167 for His inserts a bulky aromatic residue within the TM-actin interface. Because pH value that was used in our experiment was above the pKa determined for His side chain in peptides [47], the His residue is mostly in a dissociated form that is free of charge. Because the two substitutions resulted in TM movements in opposite directions, we concluded that the change in position is not solely charge-dependent. The other possible causes are discussed below.

The Lys168Glu substitution slightly shifted TM towards the inner domain of actin, which was accompanied by an increase in the proportion of the strongly bound myosin heads, even in the presence of MgAMP-PNP and MgATP, and a raised number of the switched on actin monomers at almost all steps of the ATPase cycle.

The structural explanation of the effect of Lys168Glu was suggested by the analysis of TM sequence, which revealed that four out of seven actin-binding sites

interacting with actin's Asp25 contain pairs of basic residues located within one TM chain in "f-g" positions. Among them are Arg167-Lys168 studied in this work and Arg90-Arg91. Mutations in the "g" positions of these actin-binding sites were found in myopathy patients and it was proposed that the functional significance of the "g" sites is to stabilize the TM in the closed state by maintaining a proper orientation of the "f" sites by electrostatic repulsion [18]. Effects of the Arg91Gly mutation on the orientation of the thin filament proteins and myosin heads was recently studied by polarized fluorescence method [10]. It turns out that the pattern of conformational changes during the ATPase cycle observed in the presence Arg91Gly-TM is similar to the pattern observed in the presence of Lys168Glu-TM (this work). Thus the effects can be due to direct effects of mutation in "g" position on the adjoining actin-binding residue. Indeed, both substitutions of Lys167 produced abnormal behavior of the interacting proteins.

The substitutions may also affect the helical structure of TM. According to the helix propensity scale of amino acid residues in a coiled coil peptide, Arg and Lys facilitate α-helix formation. In contrast, Gly, uncharged His and Glu have much lower helix propensity [48]. Partial unfolding of the mutant TMs was demonstrated experimentally with FASTpp method. Increased susceptibility of TMs to proteolysis with thermolysin at increasing temperatures revealed that the Arg167Gly, Arg167His and Lys168Glu substitutions destabilized TMs and decreased their melting temperatures [15]. Because destabilization of the α-helix can lead to unwinding of the coiled coil, we used COILS (www.expasy.org) to predict the effects of the mutations on the coiled coil propensity. As illustrated in Fig. 6a, the substitution of Arg167 with both Gly and His seem to have long-range effects on the probability of the coiled coil formation. Within the segment comprising Glu156 to Arg167 the probability was strongly reduced, but the effects of both substitutions were indistinguishable. In contrast, replacement of Lys168

with Glu increased the probability of the coiled coil, which is at odds with the observation of reduced thermal stability. The analysis of interactions between Lys168 and the residues in “e” position of the second TM chain shows that in the coiled coil Lys168 faces Ala166 (Fig 6b). Thus the stabilizing salt bridge is not formed and instead of being disruptive the substitution of Lys168 with oppositely charged Glu increased coiled coil propensity.

We concluded that the structural bases of the observed directions of TM movement and the consequent changes in actin and myosin heads orientation cannot be explained by simple mechanism. Most probably the conformational state of actin-TM complex depends on a combined effect of different factors, such as interfacial actin-TM electrostatic contacts, electrostatic interactions within TM’s chain and the overall shape of TM determined by stability and rigidity of the coiled coil. As shown in this and the previous work, point mutations in regions that are crucial for TM structure strongly affect the function of the actin-TM complex.

It is worth noting that in the relaxed state formed in the presence of AMP-PNP and MgATP, two mutant TMs – Arg167Gly-TM and Lys168Glu-TM, “froze” a significant fraction of myosin heads in a state that was strongly bound to actin. A similar behavior of myosin S1 was observed previously in our studies on disease-causing missense mutations in tropomyosin genes expressed in skeletal and cardiac muscles. In particular, we observed this phenomenon in case of Arg91Gly substitution in skeletal Tpm2.1 [10] and Glu180Gly and Asp175Asn substitutions in heart Tpm1.1 [49]. Together the data suggest that the altered conformation of TM-actin complex affects affinity of myosin to actin, which may restrict cycling of myosin cross-bridges.

In conclusion, the polarized fluorescence analysis of labeled actin, TM and myosin S1 reconstituted in ghost fibers showed that alterations in the TM-actin interface

by amino acid substitutions in TM have profound effects on conformation of tropomyosin, actin and myosin heads at all stages of the actomyosin ATPase cycle. In the light of the results obtained in this work, the muscle weakness in patients with congenital myopathies and reduced actin-myosin interactions observed in *in vitro* experiments can be explained by two different mechanisms: (1) blocking the filaments in the inactive state which inhibits binding of myosin to actin; (2) restriction of myosin heads cycling by "freezing" myosin heads on actin even in the weak-binding states.

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Legends to Figures

Fig. 1. Diagrams explaining the calculation of the polarized fluorescence parameters. A: The laboratory frame is taken as OXZY, where OY is the direction of propagation of incident and emitted light. OZ is the axis of the muscle fiber. θ is the angle between the actin filament axis (OW) and the fiber axis (OZ) at parallel (||) or perpendicular (⊥) direction of excitation light. B: The molecular frame for the thin filament is taken as OUVW, where OW is the filament axis. The direction of the absorption dipole A and emission dipole E for a fluorescent dye are defined by the angles Φ_A and Φ_E, respectively. γ is the angle between A and E dipoles.

Fig. 2. The effect of the Arg167His (R167H), Arg167Gly (R167G) and Lys168Glu (K168E) substitutions in Tpm1.1 on the values of Φ_E (a) and $\theta_{1/2}$ (b) of the polarized fluorescence of FITC-phallodin-actin, revealed in glycerol-skinned fibres during simulation of the sequential steps of the ATPase cycle. The leftmost entry in each panel presents the data obtained in the absence of S1. Calculations of Φ_E and $\theta_{1/2}$ values, preparation of the fibres, their composition, and the conditions of the experiments are described in Materials and Methods. The data are presented as the mean values ± standard error of the mean for 8–14 fibres for each experimental set up (see Table 1). Asterisks (*) indicate statistically insignificant differences between the wild-type and mutant TMs in the values of Φ_E and $\theta_{1/2}$. Error bars indicate ±SEM.

Fig. 3. The effect of the Arg167His (R167H), Arg167Gly (R167G) and Lys168Glu (K168E) substitutions in Tpm1.1 on the values of Φ_E (a) and $\theta_{1/2}$ (b) of the polarized fluorescence of 5-IAF linked with the mutant TMs incorporated into glycerol-skinned fibres revealed during the simulation of the sequential steps of the ATPase cycle. The leftmost entry in each panel presents the data obtained in the absence of S1. The conditions of the measurements and designations are as in Fig. 2. The data represent the mean values for 5–7 fibres for each experimental condition (see Table 2).

Fig. 4. The Φ_E angles of 5-IAF bound to the wild-type or Arg167His (R167H), Arg167Gly (R167G) and Lys168Glu (K168E) substitutions in Tpm1.1 during the ATPase cycle after correction on the rotation of actin monomers. The leftmost entry presents the data obtained in the absence of S1. The conditions of the measurements and designations are as in Fig. 2.

Fig. 5. The effect of the Arg167His (R167H), Arg167Gly (R167G) and Lys168Glu (K168E) substitutions in Tpm1.1 on the parameters Φ_E (a) and N (b) of the polarized fluorescence of 1,5-IAEDANS bound to S1 (AEDANS-S1), revealed in glycerol-skinned fibres during the simulation of the sequential steps of the ATPase cycle. The leftmost entry in each panel presents the data obtained in the absence of S1. The experimental conditions and designations are as in Fig. 2. The data represent the mean values for 5–7 fibres for each experimental condition (see Table 3).

Fig. 6. Effects of the point mutations on the probability that tropomyosin will adopt a coiled coil conformation (a). Involvement of Lys168 in the “e-g” interactions between tropomyosin chains (b). The symbols in (a) indicate: WTTM (circles), Arg167Gly-TM (squares), Arg167His-TM (triangles), Lys168Glu-TM (diamonds).

Fig. 7. Graphical summary of the effects of wild-type TM (A), Lys168Glu (B), Arg167His (C) and Arg167Gly (D) substitutions on the azimuthal position of tropomyosin (TM) on actin and the movement of actin monomer and myosin subfragment-1 (S1) at transition from weak to strong myosin-binding steps of the ATP hydrolysis cycle. TM is shown in light pink at weak and in dark pink at strong binding of S1 to actin; S1 is shown in light and dark green, at weak and strong binding, respectively. The direction of rotation of actin monomer, as well as of TM strands and S1 movement during transition from weak- to strong-binding state is shown by arrows. At transition from weak to strong binding of S1 to actin wild-type tropomyosin (WTTM) shifts to the filament center, the amount of switched on actin monomers and strongly bound myosin heads increases (A). At the strong- and weak-binding states the Lys168Glu (K168E) and Arg167Gly (R167G) mutations shift TM further towards the filament, induce strong binding of myosin heads to F-actin and switch the actin monomers on (B, D). The Arg167His (R167H) mutation results in restraining TM near the outer domain of actin throughout the ATPase cycle, inhibits the formation of the strong binding of S1 to actin and switching the actin monomers on (C).

TABLE 1. The effect of S1 and nucleotides on polarization ratios of FITC-phalloidin bound to F-actin in ghost fibres in the absence or presence of the wild-type (WTTM), or Arg167His (R167H), Lys168Glu (K168E) and Arg167Gly (R167G) mutant tropomyosins.

Nucleotide	S1	WTTM	R167H	K168E	R167G	$P_{\parallel} \pm$ SEM	$P_{\perp} \pm$ SEM
-	-	-	-	-	-	0.356± 0.002	0.163± 0.002
-	-	-	-	+	-	0.368*± 0.002	0.180± 0.003
-	-	-	-	-	+	0.389± 0.003	0.173± 0.002
-	-	+	-	-	-	0.369± 0.002	0.196± 0.002
-	-	-	+	-	-	0.370*± 0.003	0.202± 0.003
-	+	-	-	-	+	0.320± 0.002	0.226± 0.002
-	+	+	-	-	-	0.335± 0.003	0.239± 0.002
-	+	-	+	-	-	0.319± 0.002	0.235*± 0.003
-	+	-	-	+	-	0.317± 0.001	0.230± 0.002
ADP	+	+	-	-	-	0.344± 0.003	0.190± 0.002
	+	-	+	-	-	0.340*± 0.003	0.193*± 0.002
	+	-	-	+	-	0.333± 0.002	0.180± 0.002
	+	-	-	-	+	0.343*± 0.003	0.184± 0.002
AMP-PNP	+	+	-	-	-	0.345± 0.003	0.188± 0.002
	+	-	+	-	-	0.352± 0.002	0.206± 0.003
	+	-	-	+	-	0.331± 0.001	0.173± 0.001
	+	-	-	-	+	0.338± 0.002	0.180± 0.003
ATP	+	+	-	-	-	0.347± 0.003	0.182± 0.003
	+	-	+	-	-	0.348*± 0.002	0.159± 0.003
	+	-	-	+	-	0.336± 0.001	0.165± 0.002
	+	-	-	-	+	0.345*± 0.002	0.188± 0.003

$P_{\parallel}$ and $P_{\perp}$ measurements were performed as described in Materials and methods. The signs + and – mean that the values were obtained in the presence and absence of nucleotides, S1 or TMs. The number of fibres in experiments was 6-8. Error indicates ±SEM. S1, WTTM, Arg167His, Lys168Glu and Arg167Gly mutant tropomyosins and the nucleotides have pronounced effect on the values of $P_{\parallel}$ and $P_{\perp}$, indicating the changes in the conformational state of F-actin in ghost fibers ($p < 0.05$). Asterisks indicate statistically insignificant differences in the values of $P_{\parallel}$ and $P_{\perp}$ between WTTM and Arg167His, Lys168Glu or Arg167Gly mutant tropomyosins.

TABLE 2. The effect of S1 and nucleotides on polarization ratios of fluorescent probe AF bound to wild-type (WTTM), or Arg167His (R167H), Lys168Glu (K168E) and Arg167Gly (R167G) mutant tropomyosins in ghost fibres.

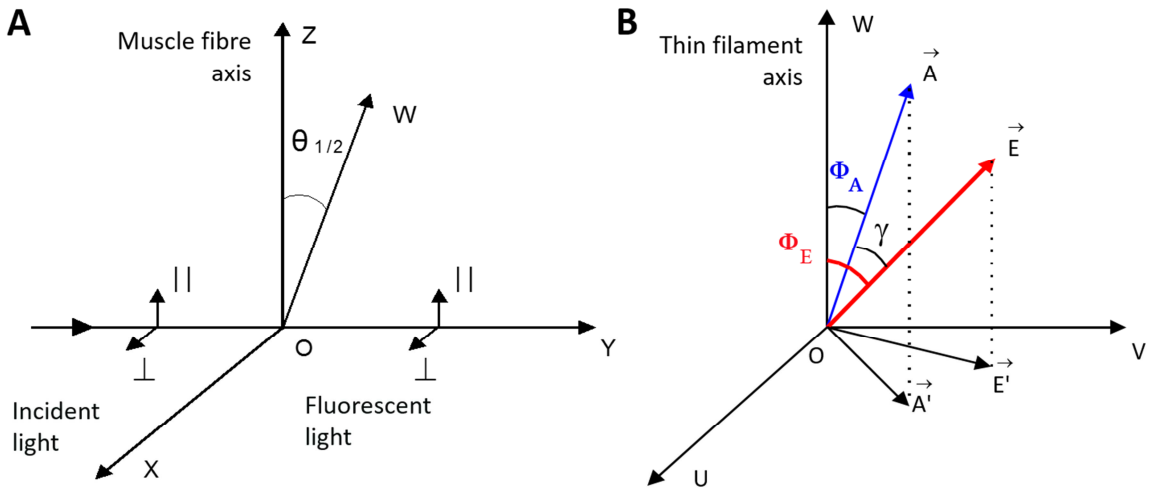
Nucleotide	S1	WTTM	R167H	K168E	R167G	$P_{\parallel}$ ± SEM	$P_{\perp}$ ± SEM
-	-	-	-	+	-	0.025± 0.001	0.129± 0.002
-	-	-	-	-	+	0.060± 0.002	0.125± 0.002
-	-	+	-	-	-	0.058± 0.002	0.167± 0.002
-	-	-	+	-	-	0.019± 0.002	0.163± 0.002
-	+	-	-	-	+	0.060± 0.002	0.125± 0.002
-	+	+	-	-	-	0.069± 0.003	0.151± 0.001
-	+	-	+	-	-	0.038± 0.002	0.144± 0.003
-	+	-	-	+	-	0.037± 0.001	0.136± 0.001
ADP	+	+	-	-	-	0.083± 0.002	0.172± 0.001
	+	-	+	-	-	0.042± 0.002	0.145± 0.002
	+	-	-	+	-	0.042± 0.001	0.123± 0.001
	+	-	-	-	+	0.033± 0.002	0.148± 0.003
AMP-PNP	+	+	-	-	-	0.060± 0.002	0.171± 0.002
	+	-	+	-	-	0.022± 0.002	0.161± 0.002
	+	-	-	+	-	0.027± 0.002	0.129± 0.002
	+	-	-	-	+	0.026± 0.002	0.142± 0.002
ATP	+	+	-	-	-	0.044 ± 0.002	0.164± 0.002
	+	-	+	-	-	-0.007± 0.002	0.151± 0.002
	+	-	-	+	-	0.007± 0.001	0.133± 0.002
	+	-	-	-	+	0.006± 0.002	0.149± 0.002

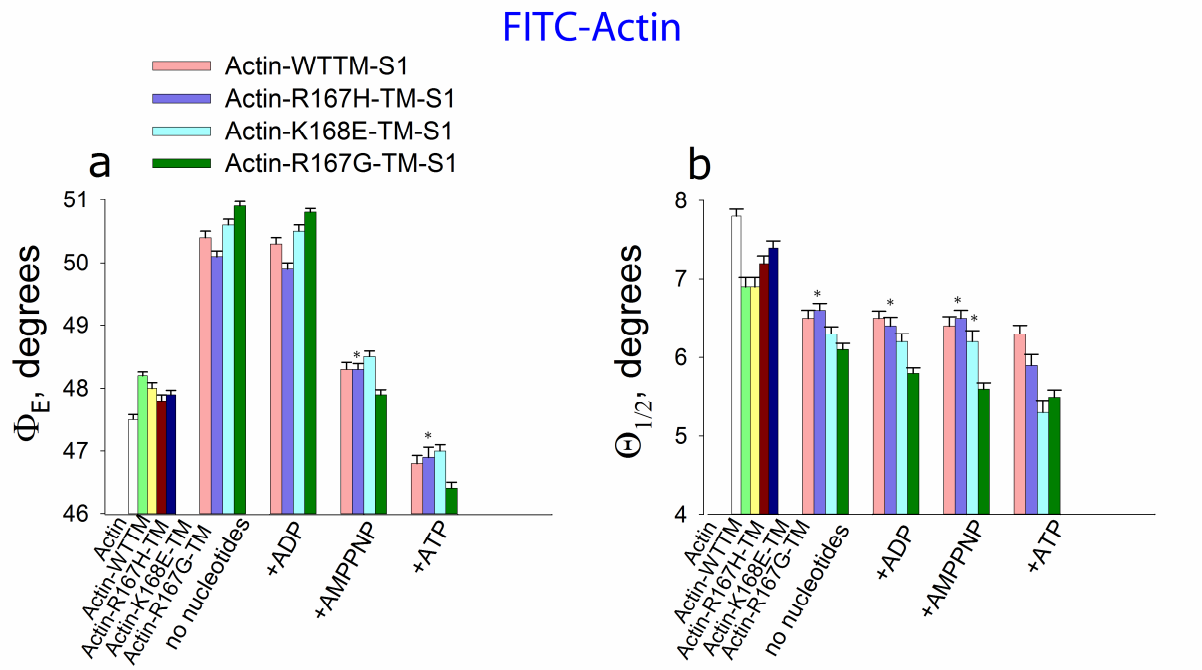
S1, WTTM, Arg167His, Lys168Glu and Arg167Gly mutant tropomyosins and the nucleotides have pronounced effect on the values of $P_{\parallel}$ and $P_{\perp}$, indicating the changes in the conformational state of TMs in ghost fibres ($p < 0.05$). Designations are as in the legend to Table 1.

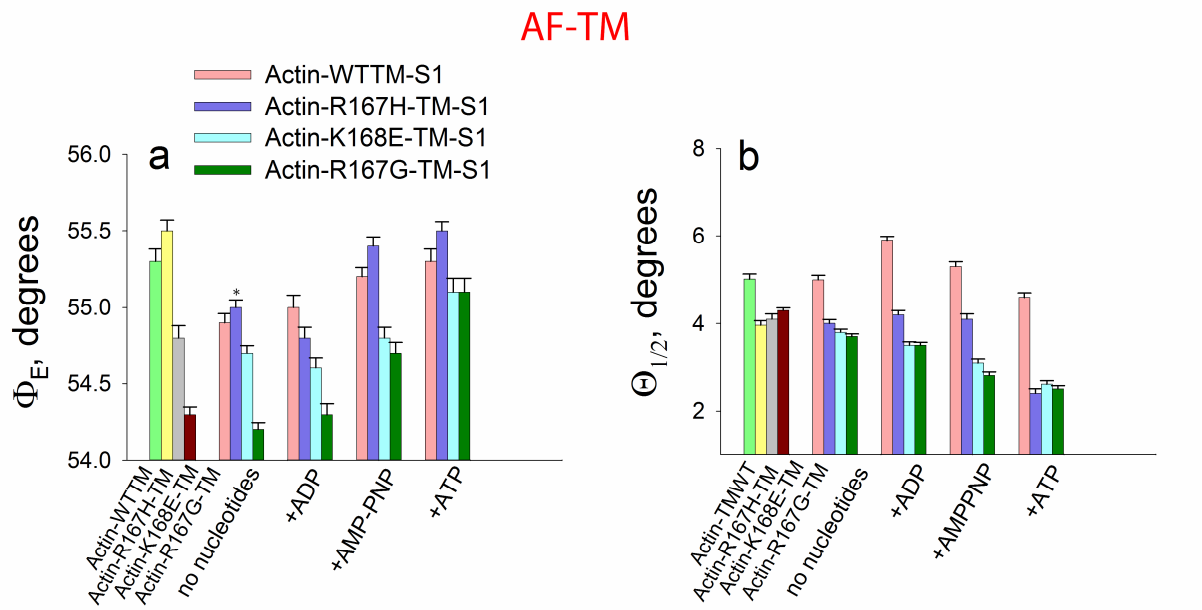
TABLE 3. The effect of the wild-type (WTTM), or Arg167His (R167H), Lys168Glu (K168E) and Arg167Gly (R167G) mutant tropomyosins and nucleotides on polarization ratios of fluorescent probe AEDANS bound to S1 in ghost fibres.

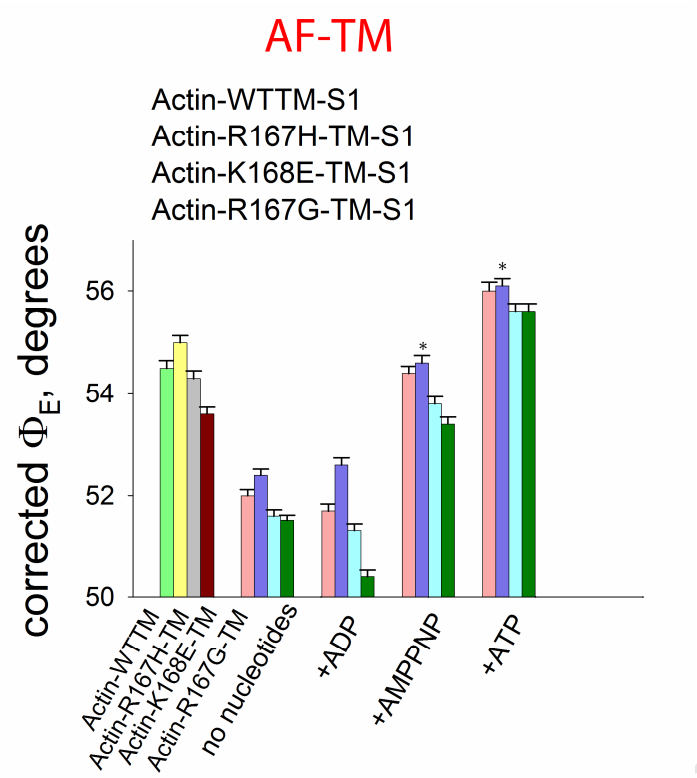
Nucleotide	S1	WT-TM	R167H	K168E	R167G	$P_{\parallel}$ ± SEM	$P_{\perp}$ ± SEM
-	+	+	-	-	-	0.376± 0.002	-0.070± 0.004
-	+	-	+	-	-	0.378± 0.003	-0.096± 0.002
-	+	-	-	+	-	0.385± 0.003	-0.127± 0.002
-	+	-	-	-	+	0.397± 0.003	-0.139± 0.002
ADP	+	+	-	-	-	0.401± 0.002	-0.041± 0.004
	+	-	+	-	-	0.405± 0.002	-0.083± 0.004
	+	-	-	+	-	0.415± 0.002	-0.104± 0.004
	+	-	-	-	+	0.418± 0.002	-0.099± 0.004
AMP-PNP	+	+	-	-	-	0.374± 0.001	0.094± 0.005
	+	-	+	-	-	0.351± 0.002	0.132± 0.004
	+	-	-	+	-	0.387± 0.002	0.045± 0.004
	+	-	-	-	+	0.399± 0.002	0.034± 0.003
ATP	+	+	-	-	-	0.332± 0.003	0.195± 0.004
	+	-	+	-	-	0.322± 0.003	0.186± 0.006
	+	-	-	+	-	0.346± 0.005	0.134± 0.006
	+	-	-	-	+	0.357± 0.003	0.127± 0.006

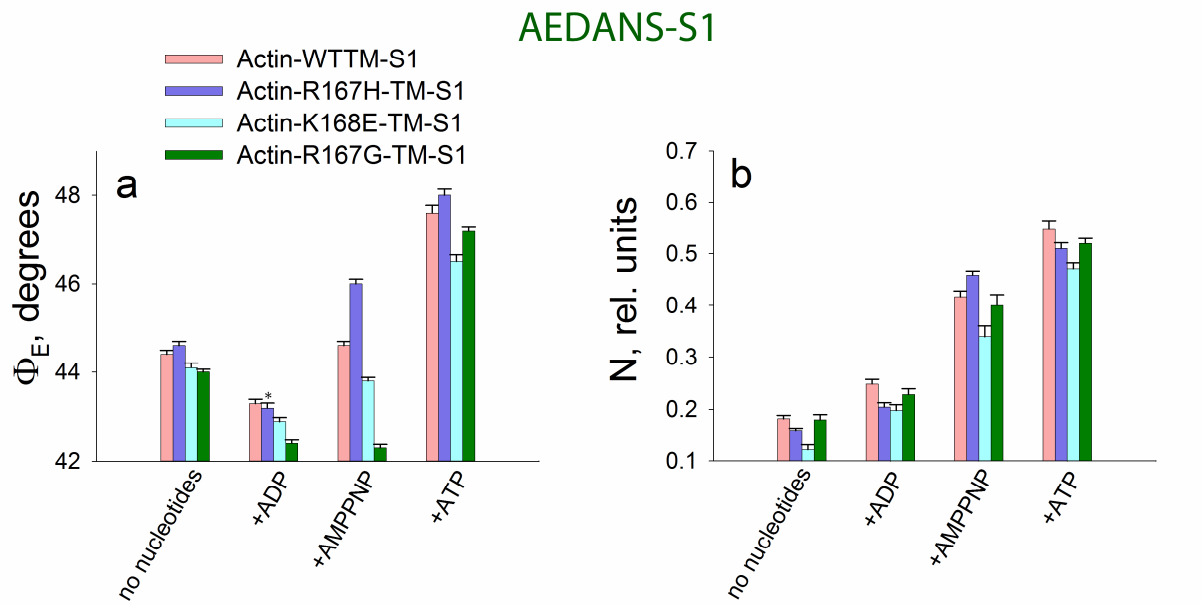
The WTTM, or Arg167His, Lys168Glu and Arg167Gly mutant tropomyosins and the nucleotides have pronounced effects on the values of $P_{\parallel}$ and $P_{\perp}$, indicating changes in the conformational state of S1 in ghost fibres ($p < 0.05$). Designations are as in the legend to Table 1.



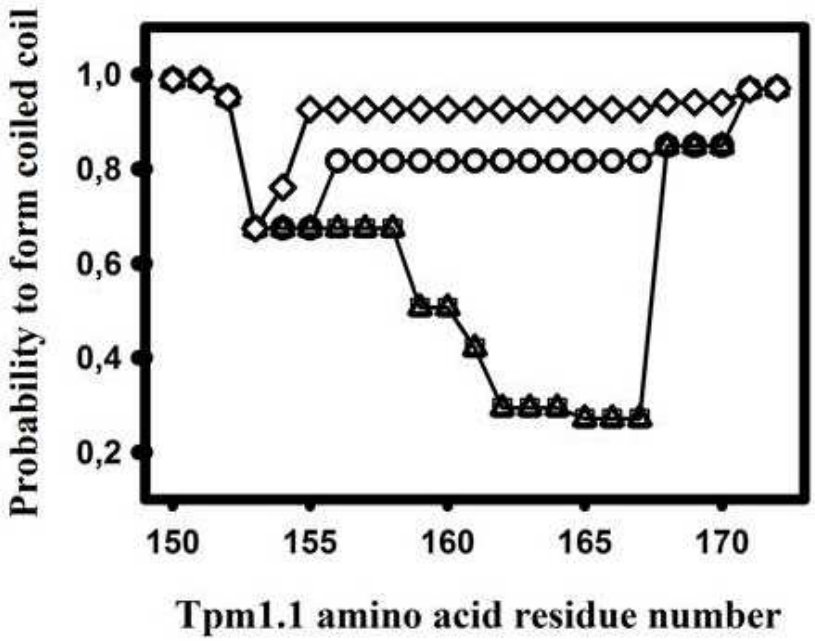




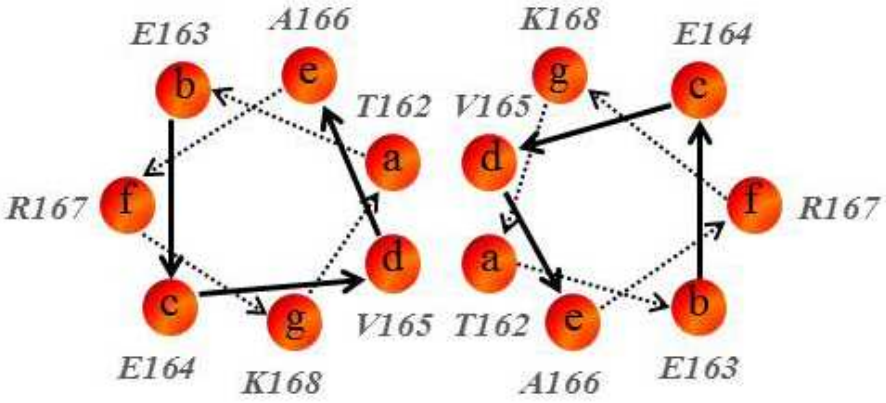




a

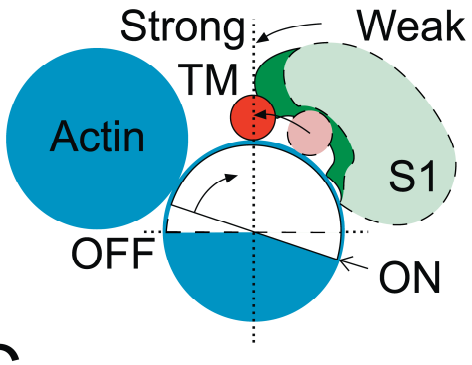

Probability to form coiled coil
1,0
0,8
0,6
0,4
0,2
150
155
160
165
170
Tpm1.1 amino acid residue number

b


E163
A166
K168
E164
T162
V165
R167
R167
V165
T162
E164
K168
A166
E163

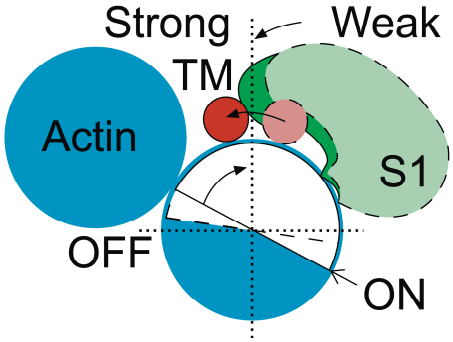
A

Actin-WT TM-S1



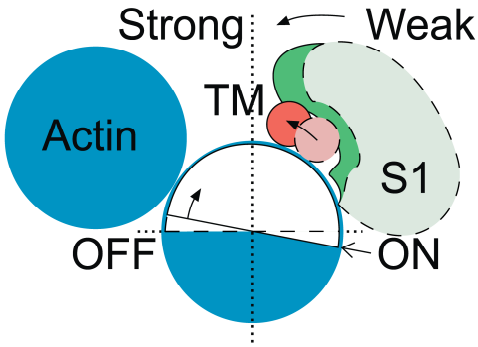
B

Actin-K168E TM-S1



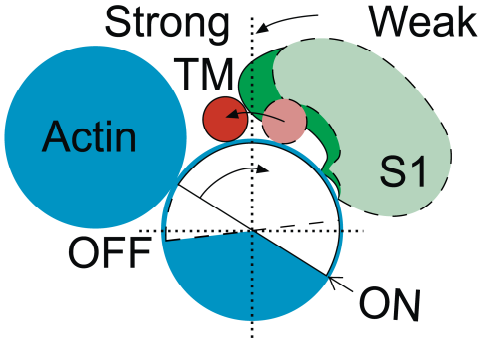
C

Actin-R167H TM-S1



D

Actin-R167G TM-S1



Highlights

- effects of Arg167His, Arg167Gly and Lys168Glu mutations in tropomyosin were studied.
- polarized fluorescence reported orientation of tropomyosin, actin and myosin heads
- mutant tropomyosins were shifted from the normal position on the actin filament
- Arg167His decreased but Arg167Gly and Lys168Glu increased the switching on of actin monomers
- Arg167Gly or Lys168Glu increased fraction of myosin bound in rigor