

The Effect of *Gly126Arg* Substitution in Alpha-Tropomyosin on Interaction of Myosin with Actin in the ATP Hydrolysis Cycle

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Received April 16, 2018

Abstract—It is known that the regulation of muscle contraction is carried out by tropomyosin and calcium-sensitive protein troponin, which form thin filament with the F-actin. The noncanonical glycine residue at position 126 of the central part of the skeletal alpha-tropomyosin destabilizes the structure of this protein. The substitution of glycine residue by arginine residue stabilizes the central region of tropomyosin, displaces tropomyosin to the open position and activates the switching actin monomers on during the ATP hydrolysis cycle. To investigate how *Gly126Arg* substitution affects the interaction of the myosin head with F-actin in the ATP hydrolysis cycle, the myosin subfragment-1 (S1) was modified with a 1,5-IAEDANS fluorescent probe and AEDANS-S1 was incorporated into the ghost muscle fiber. Multistage changes in the mobility and spatial organization of the myosin head during simulation of different stages of the ATP hydrolysis cycle were studied by polarization fluorescence microscopy. It was shown that, in the regulated thin filaments of the ghost muscle fiber at high concentrations of Ca^{2+} , *Gly126Arg* substitution significantly increases the number of myosin heads strongly associated with F-actin when simulating strong binding of myosin to actin, but reduces the number of such heads when imitating weak binding of myosin. Such changes in the behavior of myosin in the ATP hydrolysis cycle indicate an increase in the efficiency of myosin cross-bridges. A significant increase in the relative amount of myosin strongly bound to actin was also observed at low Ca^{2+} concentrations. This indicates an increase in Ca^{2+} -sensitivity of a thin filament initiated by *Gly126Arg* substitution. The obtained data suggest that the stabilization effects of the central part of tropomyosin by *Gly126Arg* substitution are realized through the abnormal behavior of tropomyosin and troponin, which leads to a change in the nature of the interaction of myosin with actin and tropomyosin in the ATP hydrolysis cycle.

Keywords: amino acid substitution in tropomyosin, actin-myosin interaction, regulation of muscle contraction, muscle fiber, polarized fluorescence

DOI: 10.1134/S1990519X1806010X

INTRODUCTION

Muscle contraction is the result of the cyclic interaction of myosin heads protruding from thick filaments with the actin of thin filaments and ATP and is carried out during sliding of thin and thick filaments relative to each other (Gordon et al., 2000). There is sufficient evidence to suggest that a thin filament consisting of F-actin and troponin–tropomyosin complex alongside is a cooperative allosteric system. Tropomyosin, troponin, and actin regulate the actin–myosin interaction in response to a change in Ca^{2+} concentration (Gordon et al., 2000; Hitchcock–Degregori,

2008). At low concentrations of Ca^{2+} subdomain-I of troponin bound with actin and tropomyosin, tropomyosin is shifted to the outer actin domain and actin monomers are switched off (i.e., the ability of the thin filament to activate the hydrolysis of ATP by myosin is inhibited) (McKillop and Geeves, 1993; Lehman et al., 1994; Borovikov et al., 2009; Chalovich, 2012). This is the so-called “off” state of the thin filament (Lehman et al., 2013; Lehman, 2017), in which tropomyosin and actin prevent strong binding of myosin cross-bridges with actin (Borovikov et al., 2009; Lehman et al., 2013) and, therefore, inhibit the hydrolysis of ATP by myosin and muscle contraction (Gordon et al., 2000).

When the intracellular Ca^{2+} concentration increases, the latter binds to the troponin subdomain-C, some actin monomers change conformation to the

Abbreviations: ATP—adenosine triphosphate, ADP—adenosine diphosphate, $\text{ATP}\gamma\text{S}$ —adenosine-5'-[γ -thio]-triphosphate, F-actin—fibrillary actin, 1,5-IAEDANS-*N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylene diamine, S1—myosin subfragment-1.

“on” state (Borovikov et al., 2009), and tropomyosin moves toward the inner domain of actin (Borovikov et al., 2009; Lehman et al., 2009; al., 2013; Lehman, 2017). This displacement opens on the actin sites of weak binding of myosin to actin, but sites of strong binding to myosin remain mostly closed (the “closed” position of tropomyosin) (Lehman et al., 2013; Lehman, 2017). When a sufficient number of myosin heads are strongly bound to actin, tropomyosin shifts even farther to the inner actin domain (in the “open” position) (Lehman et al., 2013), and the vast majority of actin monomers “switch on” (Borovikov et al., 2009), this being the so-called “on” state of a thin filament. In this state, tropomyosin completely opens the binding sites of myosin to F-actin (Lehman et al., 2013) and, together with actin (Borovikov et al., 2009), activates the hydrolysis of ATP and muscle contraction (Gordon et al., 2000). Thus, tropomyosin plays an important role in regulating the interaction of myosin with actin.

According to modern views, the tropomyosin molecule consists of two α -helices twisted into a coiled coil and its periodic interactions with the F-actin are critical for the stabilization of the thin filament and the regulation of muscle contraction (Perry, 2001). It is known that the formation of the coiled-coil tropomyosin is facilitated by the presence of consecutive seven-member repeats *a-b-c-d-e-f-g* from alternating polar and nonpolar amino acids in the polypeptide chain of the protein. Nonpolar amino acid residues are located mainly at positions *a* and *d* on the same side of the α -helix. Forming bonds with similar residues at the *a* and *d* positions of the neighboring polypeptide chain, nonpolar amino acids form a hydrophobic core of the coiled coil. Hydrophobic interactions are stabilized by ionic bonds (salt bridges), formed between charged amino acid residues located at *e* and *g* positions of heptorepeats in different chains of coiled coil. Several noncanonical residues (for example, Asp137, Tyr214, Glu218, Tyr221, Gln263, Tyr267, and Gly126), as well as Ala, Ser, and other polar and charged residues, at the interface between two chains of tropomyosin can destabilize the molecule at these points (Brown et al. 1996; Mason and Arndt, 2004). For example, Gly126 at position *g* can disrupt the α -helical structure and the interchain salt bridge. Recently, it has been shown that substitution of Gly126 by Arg dramatically reduces the proteolytic susceptibility of Arg133 residue of the skeletal muscle tropomyosin, enhances the stabilization of the central part of the tropomyosin molecule, and increases the actin-activated Mg^{2+} -ATPase activity of myosin (Nevzorov et al., 2011).

The molecular mechanisms underlying the impaired functioning of the regulatory system of muscle fibers due to various amino acid substitutions in tropomyosin have not been adequately studied. Earlier, using the method of polarization microscopy, we showed that the substitution of glycine by arginine at

position 126 of Tpm1.1 isoform of tropomyosin causes an abnormal displacement of the mutant tropomyosin to the inner domain of actin and increases the relative amount of switched on actin monomers at the simulation of the various stages of the ATPase cycle, i.e., inhibits the switching off of the thin filament (Rysev et al., 2014).

The purpose of this work was to study the response of myosin heads to the impaired functioning of thin filaments in the muscle fiber, resulting from the stabilizing substitution of the glycine residue by the arginine residue (*Gly126Arg*) in tropomyosin. It was shown for the first time that impaired ability of the regulatory system of muscle fibers to switch off the thin filament leads to an abnormal increase in the relative number of myosin heads strongly associated with actin, both at low concentrations of calcium ions (pCa 9) and at high concentrations (pCa 4). The obtained data suggest that an abnormal displacement of the mutant tropomyosin to the inner domain of actin and inhibition of the ability of troponin to switch actin monomers off can cause an increase in Ca^{2+} -sensitivity of thin filament and increase the efficiency of the myosin cross-bridges in the ATP hydrolysis cycle.

MATERIALS AND METHODS

Preparations of Proteins

Myosin from skeletal muscles of rabbit was obtained as described earlier (Margossian and Lowey, 1982). Myosin subfragment-1 (S1) was prepared by treatment of the skeletal muscle myosin with α -chymotrypsin for 20 min at 25°C (Okamoto and Sekine, 1985). Cys707 residue of S1 was modified with 1,5-IAEDANS (Borejdo and Putnam, 1977). Troponin was isolated from fast skeletal muscles of rabbit (Potter, 1982). Recombinant wild-type tropomyosin (Tpm1.1 isoform) and *Gly126Arg*-mutant tropomyosin were expressed in BL21 (DE3) cells and purified as previously described (Robinson et al., 2007). All recombinant tropomyosins had two additional amino acids (Ala, Ser) that compensated the reduced affinity of recombinant nonacetylated tropomyosin to F-actin (Monteiro et al., 1994). The purity of the obtained protein preparation was determined by electrophoresis in a polyacrylamide gel.

Preparation of Ghost Fibers

The bundles of rabbit *m. psoas* fibers were glycerinated as described earlier (Borovikov et al., 2009). Ghost fibers were prepared by incubation of single glycerinated skeletal fibers for 1.5 h in 800 mM KCl, 1 mM $MgCl_2$, 10 mM ATP, and 67 mM phosphate buffer, pH 7.0. Such fibers contained about 80% of actin and Z-line proteins. Tropomyosin, troponin, and S1 were incorporated into the F-actin filaments by the incubation of ghost fibers in a solution contain-

ing 50 mM KCl, 3 mM MgCl₂, 1 mM DTT, 6.7 mM Na,K-phosphate buffer, pH 7.0, and the corresponding protein. The unbound proteins were washed out by incubation of the fibers in the same buffer devoid of proteins. The molar ratio of WT tropomyosin or mutant tropomyosin to actin was 1 : (6.5 ± 2). In the absence of nucleotides and in the presence of ADP, ATPγS, and ATP, the molar ratios of S1 to actin were 1 : (5 ± 2), 1 : (5 ± 2), 1 : (8 ± 2), and 1 : (14 ± 2), respectively.

Measurement of Polarized Fluorescence of Muscle Fibers

The study was performed on ghost muscle fibers containing reconstructed thin filaments (Borovikov et al., 2009). S1, labeled with 1,5-IAEDANS fluorescent probe, was introduced into the fiber at 4°C. The unbound protein was removed by incubating the fibers in a solution containing 50 mM KCl, 3 mM MgCl₂, 1 mM DTT, 6.7 mM Na,K-phosphate buffer (pH 7.0) (Borovikov et al., 2009, 2017). Fluorescence was excited at 407 ± 5 nm, and the fluorescence intensity was recorded in the wavelength range 500–600 nm. The measurements were carried out in a flow chamber in the absence or presence of nucleotides (3 mM ADP, 5 mM ATPγS, or 5 mM ATP) simulating the different stages of the ATP hydrolysis cycle. In experiments with troponin, the solutions additionally contained 0.1 mM CaCl₂ or 2 mM EGTA (Borovikov et al., 2009, 2017).

The intensities of the four components of polarized fluorescence ($I_{\parallel\perp}$, $I_{\perp\perp}$, $I_{\perp\parallel}$, $I_{\parallel\parallel}$) were recorded with a polarization fluorimeter (Borovikov et al., 2004). The obtained data were processed using a model-dependent method (Tregear and Mendelson, 1975; Borovikov et al., 2009). It was assumed that the muscle fiber contains two populations of fluorophores: the population of ordered fluorophores, the absorption and emission oscillators of which are located at the angles of Φ_A and Φ_E , and the population of randomly located fluorophores N. The parameters of polarized fluorescence (Φ_E and N) were recorded during simulation of various stages of the ATPase cycle. The results were obtained for 5–11 fibers (20–55 measurements). The statistical significance of parameter changes was assessed using the Student's *t*-test for $P < 0.05$.

RESULTS

In accordance with our earlier results (Borovikov et al., 2009; Karpicheva et al., 2017), the binding of S1 modified with 1,5-IAEDANS fluorescent probe to F-actin initiates the appearance of polarized fluorescence of the ghost muscle fiber. Fluorescence analysis (see Materials and Methods) showed that angle Φ_E between the fiber axis and the emission dipole of the AEDANS probe for myosin heads associated with F-actin in the absence of tropomyosin and troponin

was 42.7°, and relative number of randomly located probes N was 0.37 rel. units ($P < 0.05$). This indicates that the probe is rigidly bound to the myosin head and the modified heads are orderly located in the muscle fibers (Borovikov et al., 2009, 2017; Karpicheva et al., 2017). The ordered arrangement of the myosin heads on actin allowed us to determine their orientation and binding to F-actin at different stages of the ATPase cycle. The absence of nucleotides mimicked the AM state of actomyosin, where A and M denote conformational states of actin and myosin heads, respectively. ADP, ATPγS, and ATP in combination with Mg²⁺ were used to simulate the intermediate structural states of actomyosin AM[^]·ADP, AM^{*}·ADP, and AM^{**}·ADP·Pi, respectively (Roopnarine and Thomas, 1996; Goody and Hofmann, 1980), where M[^], M^{*}, and M^{**} are different conformational states of the myosin head and Pi is inorganic phosphate. In the ATP hydrolysis cycle, the myosin heads in different states appear to be in rapid dynamic equilibrium (Geeves, 1991). Therefore, it is supposed that, in the simulation, each intermediate state of myosin corresponding to a certain stage of the ATPase cycle can be represented by a mixture of the above substates with predominance of one of them (Borovikov et al., 2009). It should be noted that, in our steady-state experiments, it was impossible to distinguish each substate. Therefore, we assumed that, in the absence of nucleotides or in the presence of MgADP, AM and AM[^]·ADP states are modeled in which myosin is strongly bound with actin, and, in the presence of MgATPγS or MgATP, AM^{*}·ADP and AM^{**}·ADP·Pi states of the ATPase cycle are modeled in which myosin and actin are weakly bound (Borovikov et al., 2009).

The value of angle Φ_E and the proportion of disordered fluorophores N in the presence of troponin and wild-type or mutant tropomyosin at high (pCa 4) and low (pCa 9) Ca²⁺ concentrations during simulation of various stages of the ATP hydrolysis cycle were different. Thus, the binding of wild-type tropomyosin and troponin to thin filaments decorated by AEDANS-S1 at pCa 9 led to a decrease in values Φ_E from 42.7° to 41.7°, $P < 0.05$, and N from 0.37 to 0.36 rel. units, $P < 0.05$ (Fig. 1). Such changes of Φ_E and N parameters can be interpreted as an increase in the number of myosin heads strongly bound with actin in the AM conformation (Borovikov et al., 2009, 2017; Karpicheva et al., 2017). Conversely, at low concentrations of Ca²⁺ (pCa 9), the Φ_E and N parameters increased (by 0.3° and 0.017 rel. units, respectively, $P < 0.05$, Fig. 1). Hence, the relative number of myosin heads strongly bound with actin of thin filaments (i.e., in AM conformation) decreased (Borovikov et al., 2009; 2017; Karpicheva et al., 2017).

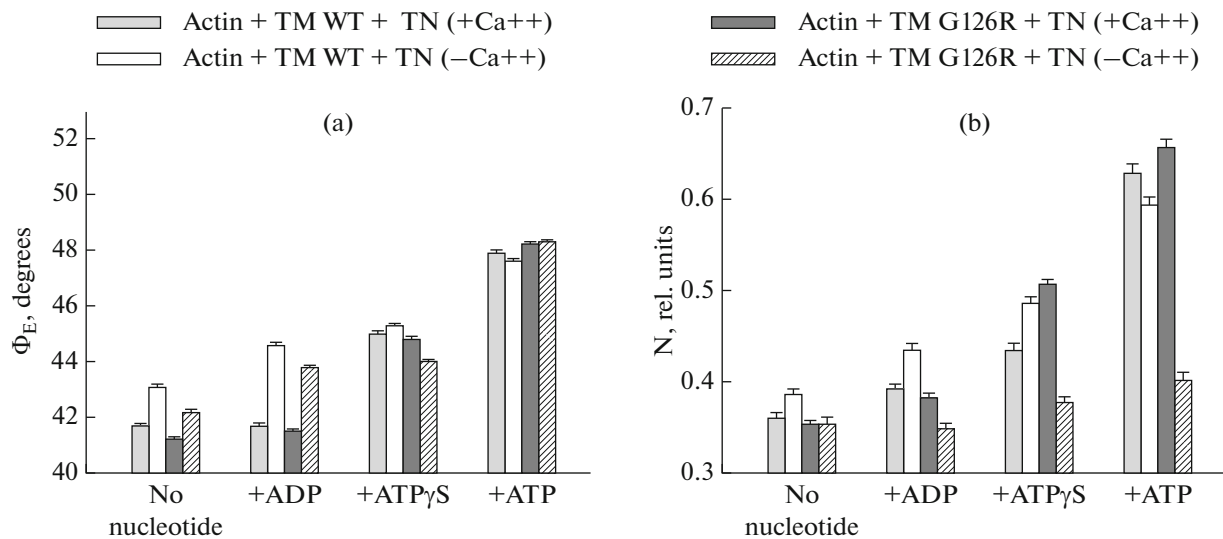


Fig. 1. Effect of *Gly126Arg* substitution in tropomyosin (isoform Tpm1.1), Ca^{2+} and troponin on the value of (a) the Φ_E angle and (b) the relative number of randomly arranged fluorophores N of the polarized fluorescence of the myosin subfragment-1 labeled with 1,5-IAEDANS in simulation of various stages of the ATP hydrolysis cycle with actomyosin. Φ_E and N parameters significantly change under the influence of amino acid substitution, concentration of Ca^{2+} , and nucleotides. All changes were significant ($P < 0.05$). Vertical segments show the standard error of the mean. Designations: TM WT, wild-type tropomyosin; G126R, tropomyosin with *Gly126Arg* substitution; TN, troponin.

As follows from Fig. 1, the replacement of wild-type tropomyosin by a mutant (*Gly126Arg*) significantly increased the number of myosin heads strongly bound with actin, both at high and low concentrations of Ca^{2+} . Such a conclusion can be drawn based on the nature of the variation of Φ_E and N parameters. It switched out that such a substitution significantly reduces the Φ_E and N values by 0.5° and 0.007 rel. units at pCa 4 and by 0.9° and 0.033 rel. units at pCa 9 ($P < 0.05$). Thus, *Gly126Arg* substitution significantly increased the relative number of myosin heads strongly bound with actin when simulating AM state of the ATPase cycle at both high and low concentrations of calcium ions. Consequently, *Gly126Arg* substitution increased the number of myosin heads in AM conformation in the presence of both high and low Ca^{2+} concentrations.

An increase in the relative number of myosin heads strongly bound to actin was also observed in the simulating of AM \cdot ADP strong-binding form and AM \cdot ADP weak binding form of the ATPase cycle stage. However, in the simulation of the weak binding form AM \cdot ADP \cdot Pi stage, no significant differences in the Φ_E parameter for experiments with wild-type and mutant tropomyosin were observed (Fig. 1a). Thus, *Gly126Arg* substitution can significantly change the nature of the interaction of myosin with actin only at some stages of the ATPase cycle: it can increase the number of myosin heads strongly bound with actin, for example, in the AM conformation. However, in

simulation of the relaxation of the muscle fiber, no noticeable changes in the orientation of the myosin heads under the influence of *Gly126Arg* substitution were observed.

It is interesting to note that, in experiments with mutant tropomyosin, the value of N for pCa 9 was significantly lower than the corresponding N values in experiments with wild-type tropomyosin (Fig. 1b). Since a decrease in the value of N indicates an increase in the binding rigidity of the myosin head with F-actin and tropomyosin (Borovikov et al., 2009), a decrease in the value of N for thin filament containing the mutant tropomyosin in the simulation of AM \cdot ADP and AM \cdot ADP \cdot Pi stages of ATPase cycle can be explained by the retention of a small number of myosin heads in the conformation of strong forms of actin–myosin interaction, for example, myosin heads in AM or AM \cdot ADP conformation (Borovikov et al., 2017).

As follows from the figure, the mutant tropomyosin increases the amplitude of the change in the Φ_E parameter when the conformation of the myosin head changes from the AM \cdot ADP \cdot Pi state to the AM state. Indeed, for the mutant tropomyosin, Φ_E decreases from 48.2° to 41.2° , $P < 0.05$, whereas for wild-type tropomyosin this parameter decreased from 47.9° to 41.7° , i.e. in the case of mutant tropomyosin, the amplitude of Φ_E changes increased by 13% compared to wild-type tropomyosin. Since it is considered that the change in the position of the SH1-helix (the probe

localization region) is transferred to the “lever-arm” (regulatory domain) of myosin, the rotation of which plays a key role in the development of the force by the myosin cross-bridge, the increase in the amplitude of SH1-helix movement in the ATPase cycle indicates that the replacement in tropomyosin increases the average statistical performance of myosin bridges (Fujita et al., 2004).

Thus, *Gly126Arg* substitution in tropomyosin increased the relative number of myosin heads in the strong-binding conformation when simulating various stages of the ATP hydrolysis cycle under both high and low concentrations of Ca^{2+} . The retention of a small number of myosin heads in AM or AM \cdot ADP conformation and an increase in the amplitude of the movement of the myosin heads (or SH1-helix of myosin) in the ATP hydrolysis cycle were detected. It is assumed that an abnormal displacement of tropomyosin to the inner actin domain and inhibition of the ability of troponin to switch on and off actin monomers (Rysev et al., 2014) can cause an increase in Ca^{2+} -sensitivity of the thin filament and increase the efficiency of the myosin cross-bridge.

DISCUSSION

It is known that tropomyosin is a coiled coil consisting of two α -helices (Hitchcock-Degregori, 2008). In a thin filament of muscle fiber, tropomyosin molecules are assembled into two continuous strands symmetrically arranged in the F-actin grooves along its entire length (Gordon et al., 2000). The tropomyosin strands are able to shift relative to the inner actin domain, depending on the concentration of Ca^{2+} and in response to binding of S1, and, in this way, to regulate the interaction of myosin with actin in the ATP hydrolysis cycle (Lehman et al., 1994, 2013; Lehman, 2017). Recently, it was shown that *Gly126* substitution with Arg in Tpm1.1 tropomyosin isoform from fast skeletal muscle causes a decrease in the flexibility of the central part of the molecule (Nevzorov et al., 2011). In addition to influencing the middle part, *Gly126Arg* substitution alters the flexibility of the C-terminus of tropomyosin. The flexibility of F-actin also changes (Rysev et al., 2014). It has been suggested that changes in the flexibility of tropomyosin and F-actin are a consequence of the conformational changes in these proteins caused by changes in electrostatic bonds between the tropomyosin strands and F-actin. It was found that *Gly126Arg* substitution induces a change in the relative position of tropomyosin and actin monomers (Rysev et al., 2014). This means that replacement causes conformational changes in tropomyosin and F-actin that can spread to regions involved in contact between these proteins. It switched out that *Gly126Arg* substitution shifts the tro-

pomyosin strands to the center of the filaments in an open position, i.e., to a position where tropomyosin and actin do not prevent the formation of strong-binding state between myosin heads and actin, which is essential for the generation of force. In addition, this mutant tropomyosin activates the switching actin monomers on at all stages of the ATPase cycle (Rysev et al., 2014). It has been suggested that the *Gly126Arg* substitution effect is due to a change in the electrostatic interaction between tropomyosin, F-actin, and myosin in the ATP hydrolysis cycle (Rysev et al., 2014).

In the present study, we found that anomalous displacement of tropomyosin to the inner domain of F-actin and an increase in the number of switched-on actin monomers in the ATPase cycle (Rysev et al., 2014) activate the formation of strong forms of binding of myosin to actin (Fig. 1). These strong-binding forms are essential for generating the force. The appearance of an abnormally high number of myosin heads strongly bound to actin under low concentrations of Ca^{2+} demonstrates an increase in the Ca^{2+} sensitivity of thin filaments (Borovikov et al., 2017).

It should be noted that the increase in Ca^{2+} -sensitivity (Shchepkin et al., 2013; Matyushenko et al., 2017) and the increase in the force developed by the myosin cross-bridges (Shchepkin et al., 2017) were also found for tropomyosin with two substitutions—*Gly126Arg* and *Cys190Ala*. Since *Cys190Ala* substitution probably did not have a significant effect on the functional properties of wild-type tropomyosin (Matyushenko et al., 2017), it can be supposed that impairments in the regulation of the actin-myosin interaction associated with *Gly126Arg* and *Cys190Ala* substitutions were mainly due to *Gly126Arg* substitution. This substitution causes abnormal behavior of tropomyosin and troponin in the ATPase cycle: “freezing” of tropomyosin near the outer actin domain and an increase in the number of activated actin monomers (Rysev et al., 2014). If, in fact, *Cys190Ala* substitution does not significantly affect the functional properties of tropomyosin (Matyushenko et al., 2017), an increase in Ca^{2+} -sensitivity of thin filaments containing *Gly126Arg* and *Cys190Ala* substitutions in tropomyosin (Shchepkin et al., 2013) and an increased force in experiments with such a mutant tropomyosin (Shchepkin et al., 2017) may be associated with an abnormal displacement of the mutant tropomyosin toward the inner actin domain and an increase in the number of activated actin monomers in the ATPase cycle.

Thus, the use of the polarization microscopy method and muscle fibers with reconstructed thin filaments allowed studying the effect of *Gly126Arg* substitution in tropomyosin on the interaction of myosin

with actin in the ATP hydrolysis cycle. It was shown that nucleotides and Ca^{2+} cause a change in the conformational state of these proteins and can disrupt the equilibrium state of the tropomyosin-troponin ensemble, thereby inducing the transition of the thin filament to the switched-on state. The results that we obtained earlier indicate that formation of the new salt bridge due to *Gly126Arg* substitution can cause the stabilization of a thin filament by inhibition of the transition of thin filament from the switched-on state to the switched-off state. This may be the reason for an increase in the Ca^{2+} sensitivity of a thin filament and activation of the formation of strong forms of myosin binding to actin in the ATP hydrolysis cycle (Fig. 1). This substitution may likely act by changing the electrostatic interaction between tropomyosin, troponin, actin, and myosin (Rysev et al., 2014).

ACKNOWLEDGMENTS

The study was supported by the Russian Science Foundation, grant 17-14-01224. The obtaining of recombinant wild-type tropomyosin and *Gly126Arg*-mutant form was carried out with the financial support of an FEBS Collaboration Scholarship.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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Translated by V. Mittova