Case Report

Journal of Bioscience & Bioengineering

# Myosin AT Pase Regulation: A Shift In Emphasis

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**Received Date:** 26 June 2023
**Accepted Date:** 20 July 2023
**Published Date:** 26 July 2023

**1. Abstract**

It has long been known that fibrous actin, under certain conditions, has its own ATPase activity. That is, the polymerization of actin monomers, which include the ATP molecule, is accompanied by the splitting of ATP and the release of inorganic phosphate. There are reasons to assume that the same property of actin is also retained when interacting with the myosin head, which has a split ATP molecule in the active center, as occurs during contraction of muscle fibers. Actin attracts the inorganic myosin phosphate to itself and then releases it into solution. The tropomyosin molecule, which rotates around its axis on the surface of the actin filament, after the activation of muscle contraction by $Ca^{2+}$, not only and even not so much removes the obstacle to the interaction of actin with myosin, but specifically activates myosin ATPase, bringing the positively charged amino acid closer to the site of interaction between the positively charged actin network and inorganic myosin phosphate. The troponin molecule, which regulates muscle contraction, serves as a Ca-sensitive lever that rotates the tropomyosin molecule properly: in the presence of $Ca^{2+}$, it enhances myosin ATPase, and in the absence of it, it inhibits one.

**2. Keywords:**
The regulation of muscle contraction, ATPase activity of myosin, actin, tropomyosin, troponin

**3. Why Does Actin Activate?**

To date the mechanism of actin-myosin interaction during contraction of skeletal muscle is generally understood. Schematically, this is the cyclic interaction of the myosin cross-bridges with the actin filaments, as was described by Lymn and Taylor [1]. Moreover, the combination in silico computational chemistry and cryo-electron microscopy of reconstituted F-actin-myosin complexes allow to offer detailed models of such interaction [2-8].

There is a consensus that the step of removing inorganic phosphate from the active site of the myosin motor domain is essential for understanding actin-activated myosin ATPase activity. The structural details of conformational changes in the motor domain that accompany the release of inorganic phosphate (back door) are discussed in several reviews [9-12]. One difficulty with this model is that this escape route may be sterically blocked when myosin is bound to actin[9]. This difficulty can be circumvented by assuming that inorganic phosphate is released towards actin rather than away from it. The same scenario has been suggested before [13]. It may be that Pi produced by ATP cleavage acts as a “door stop” in the hinge of S1, which prevents closure of the 50-kDa cleft and movement until it is lost [9]. Incidentally, one of the two pathways for the release of phosphate from the active site, namely, backdoor I path is located between switch I and switch II and guides the phosphate into the large cleft between the U50 and L50 subdomains[14]. That is, it directly indicates that phosphate moves towards the actin surface. Again, the release of phosphate through the back door towards actin leads to a situation where the release of inorganic phosphate is directly related to the actin surface, which serves as the main arena for the regulation of activity by troponin-tropomyosin complex of striated muscle. This means that now there is a connection between the release of inorganic phosphate and the activating effect of actin on myosin.

As is known, the actin filament is assembled during polymerization from actin monomers containing the ATP with $Mg^{2+}$ or $Ca^{2+}$ ions. During polymerization actin monomers hydrolyze the ATP molecules to ADP·Pi, followed by the release of Pi into the surrounding solution. It is highly likely that this ability of actin underlies the activation of myosin ATPase during muscle contraction. The process might look like this. After myosin head in the weak-binding state (M*.ADP.Pi state ) only weakly binds to actin by L50 (lower 50 kDa ) subdomain [15], further mutual attraction of the two molecules occurs due to electrostatic interactions. In the course of this attraction the 50 kDa myosin head cleft starts to close and the distance between the surfaces of two molecules decreases, increasing the strength of the interaction[3]. At this moment, the actin monomer changes from the stable A-state to the unstable R-state. The R-state is characterized by opening the cleft as a result of the propeller-like rotation of the outer actin domain with respect to the inner domain by 12°-13° [16]. All this creates conditions for the movement of inorganic phosphate from the active center of the myosin head to the cleft between the large and small subdomains of the actin monomer. The bottom of this cleft is made up of charged amino acids such as R183, K336, E72, H73, which

together create a positively charged network. One can call this part of the actin monomer a positive actin center (PAC). Inorganic phosphate leaving the active center of the myosin head enters this part of the actin monomer and is released into the surrounding solution as occurs during the polymerization of the monomeric actin-ATP complexes [17,4]. In this case, the actin monomer from the R-state returns to the stable A-state, at which the cleft closes.

Transition of actin from R-state to A-state and release of Pi occurs much faster than during actin polymerization, facilitated by conformational changes in the myosin head left without Pi in the active site. Thus, a change in the conformation of the myosin head contributes to a change in the conformation of actin, and thus myosin helps itself to be released from phosphate. In the end, it is hard to imagine what exactly prohibits actin, as a filamentous protein, from releasing phosphate in such a way that each of its monomers successfully does it. A number of facts confirm the importance of the interdomain space on actin influencing the ATPase activity of myosin. The bound $Br^8$ADP nucleotide affects the myosin S1 ATPase activation by actin; both $V_{max}$ and $K_m$ actin decreased significantly[18] probably due to the introduction of a negatively charged bromine atom into the PAC region of actin. Proteolysis studies and fluorescence resonance energy transfer assays reveal shifts in the position of subdomain 2 in ATP vs. ADP monomeric actin [19]. The possible dynamic role of subdomain 2 of actin in muscle contraction is implicated by the observations that both proteolytic cleavage of the "DNase I binding loop" and crosslinking of this loop to the C-terminus of an adjacent subunit inhibit force generation in the actomyosin interaction [20]. Formation of the R-state of the actin monomer is likely to involve opening of the back door allowing Pi to dissociate [21]. Case reports the fatal hypertrophic cardiomyopathy and nemaline myopathy associated with ACTA1 K336E mutation, amino acid lysine, which is part of the positively charged PAC network [22]. Thus, it is the creation of a local positive charge at the actin center PAC, which attracts the released by myosin inorganic phosphate, that is the mechanism of activation of myosin ATPase. Now actin transfers the arena of actions to regulate the contraction to itself.

Today the existing models of actin-myosin interaction do not reflect this state of affairs. It seems that myosin phosphate in nucleotide binding site is located too far from the acceptors on the actin surface. Our current knowledge of actin-myosin interaction is based on X-ray crystallography and on electron microscopy. Both of these techniques determine a static structure of actin-myosin complex of the strong-binding (rigor) actin-myosin interface. And the events associated with the transition of inorganic phosphate from the myosin molecule to the actin molecule occur in the time interval between the weakly bound and strongly bound states of actomyosin. And such a transition is associated with conformational transformations of both molecules, which cannot be traced by currently existing means. Only the consequences of such a transition are available in the form of existence a static structure of actin-myosin complex. It is known that actin is a very dynamic molecule and allosteric interactions with actin binding proteins are decisive to most of its functions [23].

There is evidence that the 50 kDa myosin cleft is closed by a 16˚ rotation of the U50 (upper 50 kDa) subdomain [4] which can bring phosphate closer to the outer boundary of the molecule. And if this boundary approaches the actin surface, then such interaction will become possible, if we keep in mind that the distance from the active center to the actin-binding surface initially was around 4 nm. Such an approach is even more likely due to the fact of direct contact of the light chains A1 and A2 of the myosin head with the outer domain of actin [24]. Here is evidence that the light chains A1 binds to the C-terminal part of actin at residues 360-363 [25-27]. Even if the neck of myosin head contacts the actin surface ( which is not reflected in modern models of actin-myosin interaction) why not contact the active center which would just fall in the middle of the actin monomer? Such an event is made even more probable by a fact established back in the seventies of the last century [28]. We are talking about the ability of actin to take away magnesium ions located in the active center of the myosin head, which led to the loss of the ATPase activity of myosin. This phenomenon allows us to imagine how close the active site of the myosin head and the interdomain space in actin, which contains magnesium ions, fit together.

Until now, the "active" or "inactive" state of the actin monomer in the regulation of muscle contraction was considered only indirectly, through the activity of the myosin ATPase. This model allows to characterize this state by the protein conformation. The inactive state of actin ("OFF" state) in the thin filament can be viewed as a state in which the PAC is impaired either by introducing a negative charge or by steric shielding, as in the case of actin-binding proteins. Accordingly, the active state ("ON" state) occurs when the PAC is restored.

### 4. How Does Tropomyosin Regulate The Actomyosin Cycle?

Tropomyosin (Tm), an extended coiled-coil dimer, binds end-to-end along the actin filament and covers seven actin monomers. The most widely cited model for Tm mediated regulation of actin-myosin activity assumes that regulated thin filaments may exist in three biochemically distinguishable states: the "blocked"state, where Tm sterically blocks specific myosin binding to actin, the "closed" state, which permits weak specific binding, and the "open" state, in which strong rigor-like actomyosin cross-bridges may form [29-32]. It is known that Tm has a low affinity of binding to actin and therefore is easily displaced by the myosin head from its position on the actin filament. $Ca^{2+}$-free troponin (Tn) increases the affinity of the interaction of Tm with the actin by about a hundred times [33]. But even this strengthening of the bond does not allow Tm to keep the onslaught of myosin, and at a high concentration of the myosin heads, the rate of the ATPase becomes almost the same as that of an unregulated actin filaments [34]. In other words, the blocking properties of Tm in conjunction with Tn become apparent only in the absence of $Ca^{2+}$- and only at a low concentration of myosin heads, as is the case in a state of relaxation, when the degree of overlap of actin and myosin filaments is minimal. Experiments in vitro show that myosin subfragment 1 (S1) does bind to thin filaments in the absence of $Ca^{2+}$- but appears unable to complete the cycle of interaction [35].

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Suggested model assume that with the flux of $Ca^{2+}$ into the muscle fibre, Tn rotates Tm in such a way that tropomyosin rolls by an azimuthal rotation of about 16° and brings the positively charged amino acids closer to the actin PAC,

whereas before its rotation Tm creates a negatively charged or non-polar field next to the PAC of the actin. Due to this, tropomyosin accelerates the ATPase of actin-myosin during muscle contraction. Further in this section, evidence will be given to confirm the correctness of this assumption. The idea that Tm "rolls", not "slides" across the actin surface, is originally proposed by McLachlan and Stewart [36]. In this model Tm's α-band residues interact with the actin surface in the absence of $Ca^{2+}$, whereas β-band residues move toward actin in the presence of calcium. The data of other authors are consistent with this model [37,38].

Now, if we return to the problem of regulating the ATPase cycle, the process of the actin-myosin interaction will look like this. In the relaxed state (without $Ca^{2+}$) the M-ADP-Pi weak state rebinding to actin in inactive state, being driven through the positively charged loop 2 (connecting the U50 and L50 domains), interacting with the negative charge of the N-terminus of actin. Tm located near outer domain of actin with the support of Tn, does not allow (either sterically, or allosterically, or by a combination of these two ways), myosin head to crawl over the actin monomer. In such prepowerstroke state, the myosin head remains until $Ca^{2+}$ begins to flow. Under the influence of calcium on Tn, the latter begins to shift Tm towards the center of the actin axis, and myosin is able to complete its attachment to the actin. The first stage could involve the formation of stereo-specific hydrophobic interactions between actin and the L50 domain. The second step is followed by 50 kDa cleft closure and formation of additional interactions between actin and the U50 domain with the formation of the complete actomyosin contact surface (strongly bound rigor actin–myosin interface). The 50 kDa cleft closure accompanied by the displacement of a large volume of bound water from the actin-myosin complex, produces the conditions at which phosphate presumably attracts to the positively charged PAC of actin. Simultaneously with the advancement of the motor domain towards the center of the actin filament, Tm turns over and at the moment when the actin PAC approaches the nucleotide-binding site of myosin as much as possible, Tm completes its turn and strengthens the positively charged field near the PAC. Phosphate is released. The loss of Pi is associated with relieving the strain in the upper 50K β -sheet and the swing of the converter and lever – arm. These events are likely to have equivalent processes in the pre-powerstroke to rigor transition. Thus, this interpretation of events is more suitable for the concept of a two-state model for Tm-mediated regulation of actin-myosin activity: inactive state at which the ATPase is significantly slowed down and active at which the ATPase works at a suitable speed [39,40].

Recently, both EM reconstruction and fiber diffraction studies of reconstituted F-actin-tropomyosin have revealed the azimuthal position and the longitudinal (z)-positioning of tropomyosin molecules on actin subunits along the axial length of the filaments [41,42]. And now we have the opportunity to compare which amino acids on the actin surface will interact with amino acids on the tropomyosin surface when tropomyosin moves from a closed state to an open one using the example of the 5th quasi-repeat of α-tropomyosin. As follows from studies reported in Li et al. [42], in closed state the α-band of the 5th quasi-repeat of α-Tm covers outer domain of the actin. Wherein the K326 and K328 residues of actin approach E181 and E177 residues of tropomyosin, respectively, actin's P333 residue comes to V170 residue on tropomyosin, actin D25 residue goes closer to R167 on tropomyosin and K336 residue (PAC) on actin approaches the E163 residue on tropomyosin (Fig. 1, 3). Thus, the negatively charged tropomyosin's E163 residue is located above the region of the PAC in actin, inhibiting the work of the ATPase in the closed state of tropomyosin. In the 4th quasi-repeat α-band of Tm, the PAC region of actin is in contact with E124, and in the 6th with T199, for example.

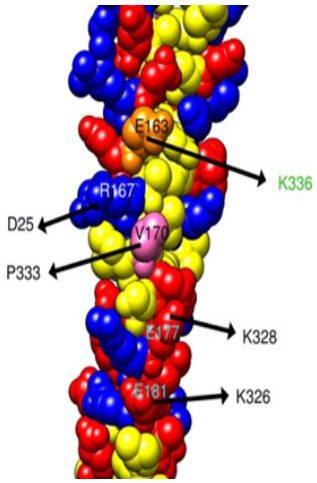


**Fig 1:** The 5th quasi-repeat α-band of α-Tm (PDB 2B9C ).

The tropomyosin strands point toward the barbed end of the thin filament and a face-on view of the Tm- F-actin interface is shown in closed state (at low Ca2+). The arrows move away from residues on tropomyosin and point to residues of actin with whom they interact. Basic amino acids are painted blue, acidic amino acids are red. The residue V170 (hot pink) points to actin Pro-333, acidic E163 (orange) points to actin PAC (residue K336, green). The visualization system Chimera was used to prepare the figures [43].

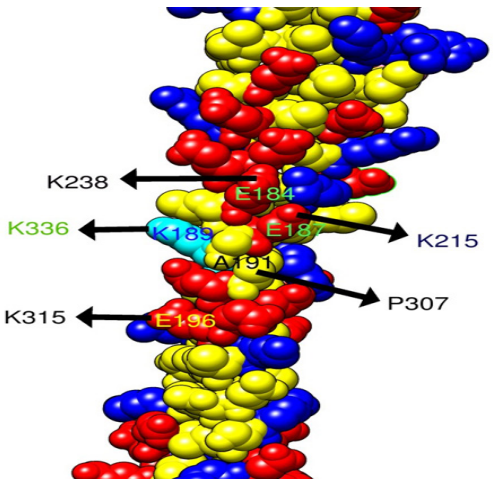

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**Fig 2:** The 5th quasi-repeat β-band of α-Tm (PDB 2B9C ).

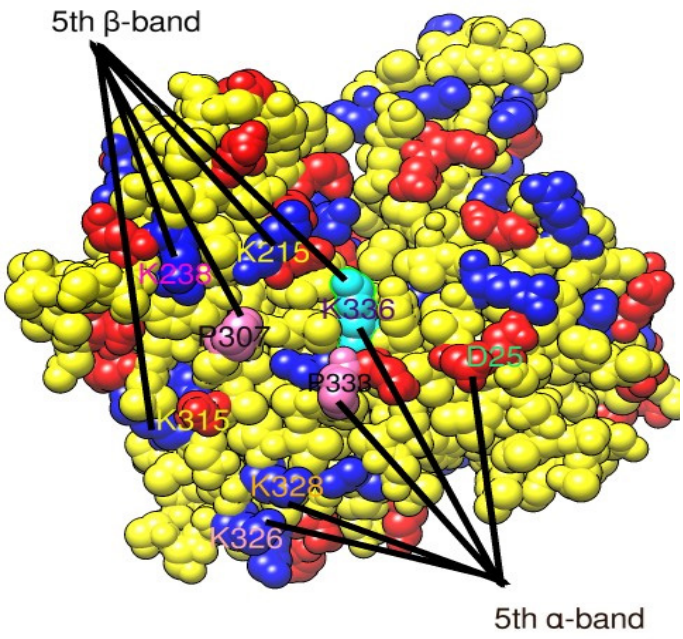


**Fig 3:** Location of amino acids on the surface of actin interacting with amino acids of tropomyosin in the closed state (α-band) and open state (β-band) of the actin. Sphere representation of uncomplexed actin (PDB 1j6z). Basic amino acids are painted blue, acidic amino acids are red. The residues Pro-333 and Pro-307 are shown in hot pink. The residue K336 (cyan) of positive actin center interacts with tropomyosin both in the closed state and in the open state of actin.

The tropomyosin face-on view of the Tm- F-actin interface is shown in the open state (plus $Ca^{2+}$). The arrows move away from residues on tropomyosin and point to residues of actin with whom they interact. Basic amino acids are painted blue, acidic amino acids are red. The basic residue K189 (cyan) points to actin PAC (residue K336, green).

Julian von der Ecken [44] using a direct electron detector and drift correction have determined the structure of F-actin in complex with tropomyosin. From their data it follows that rolling of Tm would imply not only an azimuthal shift of ~12 Å, but also a tremendous shift of a half-tropomyosin repeat along the F-actin filament. In other words, upon the transition of Tm to the open state, next to the actin PAC, the 5th quasi-repeat β-band of tropomyosin, covering the inner actin domain, will appear. Wherein K315 residue of actin approaching E196 on tropomyosin, P307 on actin approaching A191 on tropomyosin; actin's residues K215 and K238 are close to E187 and E184 on tropomyosin, respectively and K336 (PAC) on actin is close to K189 on tropomyosin (Fig. 2, 3). Thus, the positively charged K189 tropomyosin residue is located nearby the actin region of the PAC, accelerating the work of ATPase in the open state of tropomyosin. In the third quasi-repeat β-band of α-tropomyosin the PAC region of actin is in contact with K112 and in the 4th with K152, for example.

It follows that Tm in striated muscle plays a more active role than the steric blocking theory suggests: tropomyosin not only blocks and then moves to allow actin to interact with myosin, but also activates actin-myosin ATPase itself when calcium ions enter during muscle contraction. It has long been noted that Tm is able to accelerate myosin ATPase but this property was attributed to the cooperative effect. The concept of a cooperative effect appeared in connection with the study of the binding of tropomyosin to actin. It turned out that at first the process is slow, but as the first attached molecules appear, the rate of attachment of the remaining molecules increases.

The cooperative effect of ATPase upregulation was explained by the fact that Tm stabilization in the open position allows the activation of additional actins in this and, possibly, neighboring A7TmTn regulatory units. It's right. But how can one explain the fact that in the presence of Tm in thin filaments, the ATPase rate exceeds the ATPase rate of unregulated actin-myosin, where all myosin-binding sites of actin are initially open? And there is no need to open and activate neighboring actin molecules? The assumption that Tm enhances the positively charged field and thereby accelerates the ATPase, it is thus easy to imagine. If we assume that Tm touched actin with a "negative" surface before rotation, then at the end of rotation on 70 degree , Tm contacts actin with a "positive" surface. If the main function of tropomyosin in the muscle fiber is to prevent actin from interacting with myosin, as the steric blocking hypothesis insists, then any "log" would be suitable for this purpose. However, tropomyosin has a unique composition that is not repeated in any other protein; at each turn, an area saturated with negatively charged amino acids ("negative" surface) alternates with a "positive" surface area [36]. And why did tropomyosin need to roll over at all? For blocking, a simple displacement of the molecule to the center of the actin filament would be suitable.

This model of interactions also makes it possible to explain the S-shaped dependence of the calcium sensitivity of actin-myosin ATPase on the concentration of free calcium. With an increase in low $Ca^{2+}$ concentrations (pCa 9–7.5), ATPase activity is low and the dependence curve runs almost parallel to the x axis. In the range from 7.5 to 6, the dependence graph rises almost in direct proportion to the maximum values. Above pCa 6, the ATPase values do not change and the graph again runs parallel to the x-axis. This phenomenon is explained by the rotation of tropomyosin around its axis, in which a positively charged amino acid approaches the PAC on actin. At low $Ca^{2+}$ concentrations, tropomyosin rotates through a small angle and the amino acid does not yet entered into the electrostatic field of the PAC, and therefore the ATPase values do not change. In the range of pCa from 7.5 to 6, the amino acid already enters this field, and even a slight rotation of tropomyosin already noticeably increases the positively charged PAC field, which is expressed in an increase in the ATPase rate. The maximum approach of the amino acid with the PAC occurs at the completion of the turn, when the maximum ATPase rate is noted. A further increase in $Ca^{2+}$ concentration from pCa 6 and above does not lead to further approach, and the ATPase rate remains maximum throughout this interval.

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**5. Role Of Troponin In The Regulation Of Muscle Contraction**

Troponin, together with tropomyosin, forms a system that regulates skeletal muscle contraction and relaxation at the molecular level. Troponin consists of three subunits: TnC, the $Ca^{2+}$-binding subunit; TnI, the inhibitory subunit; and TnT, the tropomyosin-binding subunit. Troponin appears to have two distinct functions; at low $Ca^{2+}$, troponin operates as an inhibitor, while at high $Ca^{2+}$, it acts as an initiator of the contraction. The inhibitory role of troponin is carried out by the C-terminus of TnI (residues 163 to 210) that is seen crossing the cleft between azimuthally neighboring actin monomers [45] and could be described by a "fly-casting mechanism". The activating role of troponin is imparted by TnC. Binding of $Ca^{2+}$ to the regulatory sites of N terminal domain of TnC would induce the widening of two EF hands (AB and CD) that move away from each other and the angle between helix A and B changes from 81° to 135°[46]. Wherein, $Ca^{2+}$ binding would induce opening of the hydrophobic pocket in the N-terminus of TnC and the TnI switch segment (residues 149 to 164) would bind there [47]. At the same time the central helix of TnC and the TnI inhibitory segment are significantly stabilized [47].

In [48], the authors visualized the location troponin by determining the three-dimensional structure of thin filaments from electron cryo-micrographs. The analysis showed that at high $Ca^{2+}$, the peak of the electron density of the V block was located in front of the inner actin domain and shifted towards the outer domain by 28 Å or 40 deg. with low $Ca^{2+}$. The N-terminal domain of troponin C practically did not move much, whereas its C-terminal domain shifted by 50 Å to the outer domain of actin at low $Ca^{2+}$. Therefore, TnC acted as a lever arm, and N-TnC was its fulcrum. In general, the Ca2+-induced movement of TnC appeared to be a counter-clockwise rotation from the first actin subdomain towards the fourth one [48]. This is exactly the direction of movement of the core part of troponin, which ensures the rotation and advancement of the tropomyosin cord by half of the quasi-repeat (from the α-zone to the β-zone) towards the pointed end of the actin thin filament (Fig. 4), described in the second section of this article about the role of tropomyosin.

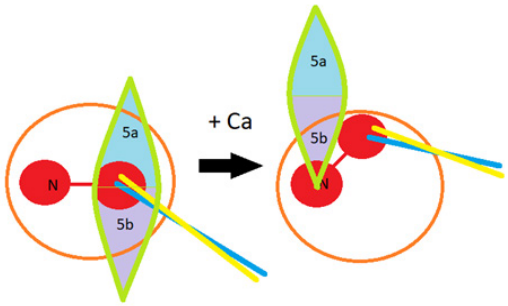


**Fig 4:** Schematic representation of the movement of tropomyosin by the core of troponin under the influence of Ca2+

The actin monomer is represented by an orange circle. The troponin core is represented by a red dumbbell-shaped TnC and a yellow-blue IT arm (TnI + TnT). The fifth quasi-repeat of tropomyosin is presented as a green diamond, where the 5α-zone is colored blue, and the 5β-violet. Under the action of Ca2+ ions, the C-terminus of TnC, together with the IT-arm, rotates around the N-terminus of TnC counterclockwise from the first actin subdomain to the fourth one [48], moving tropomyosin from the outer actin domain to the inner one, moving tropomyosin half a quasi-repeat forward to the side the pointed end of the actin filament, changing the 5α-zone in the vicinity of the PAC to the 5β-zone.

Thus, according to this model, the activating effect of troponin on the cyclic work of cross-bridges consists in the precise and coordinated movement of Tm (rotation plus move), the purpose of which is to bring the positive charge closer to the PAC of actin. Considering the contraction and relaxation of the muscle, one must not forget that two cyclic processes occur in this case: the cyclic movement of actomyosin cross-bridges and the cyclic movement of the Tn-Tm complex, while the first is preceded by the second with an interval of 12-17 msec [49]. At the same time, it should be borne in mind that the cyclic nature of actomyosin bridges includes not only the alternating attachment-detachment of myosin heads with actin, but also the cyclic nature of the movement of the myosin head over the actin surface. The myosin head moves cyclically from the outer edge of the first domain to the border between the outer and inner actin domains, as shown in [4].

During this movement, the myosin head is forced to push the Tn-Tm complex in front of it if there is no $Ca^{2+}$ in the medium. This inhibitory effect of the passive Tn-Tm complex is expressed in the slowing down of the ATPase rate, which is clearly seen from work [34]. As a result of the shift in focus from the inorganic phosphate exit through the «back door» to the active uptake of the inorganic phosphate by PAC on actin, the active role of actin is asserted. Instead of passive displacement of tropomyosin for the release of myosin-binding sites of actin, offered targeted and precise movement, leading to the activation of the ATPase. Instead of opening a hydrophobic pocket on N-TnC for TnI attachment and thus eliminating the inhibitory effect of TnI as the main event in troponin activity, a dynamic picture of the movement of the core part of troponin is proposed, which ensures the movement of tropomyosin along the actin filament, aimed at regulating the ATPase cycle.

**Acknowledgments**

The author would like to thank Dr S.V. Avrova for her review of the manuscript.

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