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Gly126Arg substitution causes anomalous behaviour of α-skeletal and β-smooth tropomyosins during the ATPase cycle

Nikita A. Rysev [a], Ilya A. Nevzorov [d], Stanislava V. Avrova [a], Olga E. Karpicheva [a], Charles S. Redwood [c], Dmitrii I. Levitsky [b], Yurii S. Borovikov [a,*]

[a] Laboratory of Mechanisms of Cell Motility, Institute of Cytology, Russian Academy of Sciences, 4 Tikhoretsky Avenue, St. Petersburg 194064, Russia
[b] A.N. Bach Institute of Biochemistry, Russian Academy of Sciences, Moscow 119071, Russia
[c] Department of Cardiovascular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, UK
[d] Institute of Biotechnology, University of Helsinki, Viikinkaari 9, 00014 Helsinki, Finland

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ABSTRACT

To investigate how TM stabilization induced by the Gly126Arg mutation in skeletal α-TM or in smooth muscle β-TM affects the flexibility of TMs and their position on troponin-free thin filaments, we labelled the recombinant wild type and mutant TMs with 5-IAF and F-actin with FITC-phalloidin, incorporated them into ghost muscle fibres and studied polarized fluorescence at different stages of the ATPase cycle. It has been shown that in the myosin-and troponin-free filaments the Gly126Arg mutation causes a shift of TM strands towards the outer domain of actin, reduces the number of switched on actin monomers and decreases and increases the rigidity of the C- and N-termini of α- and β-TMs, respectively. The binding of myosin subfragment-1 to the filaments shifted the wild type TMs towards the inner domain of actin, decreased the flexibility of both terminal parts of TMs, and increased the number of switched on actin monomers. Multistep alterations in the position of α- and β-TMs and actin monomers in the filaments and in the flexibility of TMs and F-actin during the ATPase cycle were observed. The Gly126Arg mutation uncouples a correlation between the position of TM and the number of the switched on actin monomers in the filaments.

Introduction

Muscle contraction results from the ATP-powered cyclic interaction between myosin heads protruding from the thick filaments and actin subunits in the thin filaments, causing the thick and thin filaments to slide past each other (for a review, see [1,2]). Previous studies have shown that tropomyosin (TM[1]) wraps around the thin filaments and is located in a position to influence the actin–myosin interaction. It has been suggested that TM can regulate this interaction by moving from a position where it blocks the actin–myosin interaction to the one where it allows myosin head binding to F-actin. According to this hypothesis, actin exists in two states: that of blocking myosin binding and open to it. The reduction in binding of S1 to actin by tropomyosin directly results in decreased ATPase activity (for a review, see [3]). However, subsequent biochemical and structural studies have shown that the mechanism of regulation of the actin–myosin interaction is more complicated (for a review, see [2,4]).

According to current views, regulation of striated muscle contraction involves changes that occur in the co-operative system consisting of myosin, actin, troponin and tropomyosin. A three-state allosteric model was proposed to describe this process, where S1 and Ca^{2+} are allosteric effectors of tropomyosin [5–9]. In the absence of Ca^{2+}, troponin constrains TM, which occupies a position on the outer domain of actin that sterically inhibits the binding of myosin cross-bridges to actin ("blocked position") and, consequently, the ATPase and filament sliding. Ca^{2+} binding to troponin shifts TM strands towards the inner domain of actin, exposing most of the myosin-binding sites [6–9]. However, TM still covers a part of the myosin-binding site ("closed position"). Only when myosin head attaches to the actin filament, TM moves to the inner domain of actin and fully exposes the myosin binding site on actin ("open position") [9,10]. These three structural states are in rapid equilibrium with each other [11], so that in each condition there is a distribution of states [12].

Also, a significant role of the conformational changes of actin in this regulation was revealed ([13–15] for see, for example). It was suggested that for each TM position there was a particular ratio of monomers in two states – the so-called "switched on" and "switched off" states, which differ in monomer orientation relative to the filament axis [14,15]. The switched on monomers are able,

* Corresponding author. Fax: +7 812 2970341.
E-mail address: borovikov@incras.ru (Y.S. Borovikov).

[1] *Abbreviations used:* TM, tropomyosin; WT-TM, wild type tropomyosin; TN, troponin; S1, myosin subfragment 1; FITC-phalloidin, fluorescein phalloidin; 5-IAF, 5-iodoacetamidofluorescein; DTT, dithiothreitol.

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whereas the switched off monomers are unable, to activate the ATPase, i.e. actin monomers may be in the so-called "active/inactive" functional states (for a review, see [2,4,16]).

Electron microscopy and molecular dynamics simulations show that TM is a semirigid structure which is stiff enough to move across the surface of actin as a unit when perturbed mechanically by other actin-binding proteins such as myosin [17–22]. TM is a two-chain α-helical coiled coil whose periodic interactions with the F-actin helix are critical for thin filament stabilization and the regulation of muscle contraction. Like all coiled-coils, each α-helical chain of TM displays a seven amino acid long "heptad" periodicity required to build the characteristic "knobs into holes" structure at the interface between the two adjoining α-helices [24]. In this pattern, the residues in the heptad repeat are labeled a–g. The a and d residues are hydrophobic and pack at the interface, while the e and g residues are oppositely charged and stabilize the coiled coil through interchain electrostatic interactions. Residues in the b, c and f positions are available for binding to other proteins [25].

Several non-canonical residues (e.g. Asp-137, Tyr-214, Glu-218, Tyr-221, Gln-263, Tyr-267 and Gly126) as well as Ala, Ser, and other polar and charged residues in the interface of TM can destabilize the molecule at these points [26,27]. For example, Gly126 in the g position may perturb coiled-coil structure by disrupting the α-helical structure and inter-chain salt bridge. Recently it has been shown that, like in the case with the Leu substitution at Asp 137, the mutation of Gly 126 to Ala or Arg also dramatically reduces proteolytic susceptibility at Arg-133 in both smooth and skeletal muscle TMs, increase the stabilization of the middle part of the TM molecule and ATPase activity [28].

This article presents the first study that aims to examine the effect of substitution of Gly126 with an Arg residue in skeletal α-TM or in smooth muscle β-TM on the position and flexibility of C- and N-termini of TMs on the troponin-free thin filament at different stages of the ATPase cycle using polarized fluorimetry, a technique we have previously used to study mutant TMs [29–31]. In the myosin- and troponin-free filaments both mutant TMs are found to be shifted further towards the periphery of the filaments, with the flexibility of the C-terminus of α-TM increasing and the flexibility of the N-terminus of β-TM decreasing. The binding of S1 to F-actin moves the wild-type TMs towards the center of the filaments (towards the "open position"), which results in decreased flexibility of the C-terminus of α-TM and N-terminus of β-TM and a pronounced rotation of actin monomers to the periphery of the filaments. The latter indicates an increase in the number of switched on actin monomers. The Gly126Arg mutation alters the effect of S1binding by shifting α- and β-TM strands further to the inner domain of actin, increases the flexibility of both TMs and increases the number of switched on monomers in experiments with α-skeletal TMs while leaving the number of switched on monomers in experiments with β-TMs unchanged. Multistep alterations in the position of α- and β-TMs and flexibility of TMs and F-actin during the ATPase cycle were observed. The position and flexibility of TM and orientation of actin monomers depend on the intermediate S1 state, the Gly126Arg mutation and TM isoforms. We suggest that the observed increased ATPase activity induced by substitution of Gly126 with an Arg in TM [28] may result from the uncoupling of correlation between the position of TM and the number of switched on monomers during the ATPase cycle.

Materials and methods

Preparation of proteins

All of the TMs used in this work were recombinant human proteins that have Ala-Ser N-terminal extension [32] to imitate naturally occurring N-terminal acetylation of native TM. The wile-type (WT) and mutant TMs were prepared in the bacterial expression plasmid pMW172 [33] by PCR-mediated site-directed mutagenesis using AccuPrimeTM Pfx DNA Polymerase (Invitrogen). The oligonucleotides used for mutagenesis for Gly126Arg were: GAGTGAGAGACGCATGAAAG (mutant codon is underlined). The PCR products were cloned and sequenced to verify the substitutions. The pMW172 constructs were used to transform the *Escherichia coli* strain BL21(DE3)pLysS, and large scale cultures were grown, and overexpression was induced according to standard methods [34]. Bacterial cell lysates containing recombinant Ala-Ser TMs were heated to 85 °C before clarification by centrifugation at 33,200×*g* for 10 min. The resulting supernatant was fractionated by reducing the pH to 4.8, and the recombinant protein was purified by anion exchange chromatography. The mutant TM was purified by the same method as was used for the wild-type protein. TM concentration was determined using BCA Protein Assay (Thermo Scientific). Labelling of TM with 5-iodoacetamide fluorescein (5-IAF) at Cys190 or Cys36 was performed as previously described giving probe to protein ratio 0.8:1 [35].

Myosin subfragment-1 (S1) was prepared by treatment of skeletal muscle myosin with α-chymotrypsin for 10 min at 25 °C [36]. Purity of the protein preparations, as well as the composition of the fibres after washing out of the unbound proteins, was monitored by SDS–PAGE. Protein concentrations were determined by measuring UV absorbance.

Preparation and labeling of ghost fibers

Glycerinated muscle fibers were obtained from rabbit psoas muscles by the method of Szent-Gyorgyi [37]. Ghost fibers were prepared by incubation of single glycerinated fibers for 1.5 h in the solution containing 800 mM KCl, 1 mM $MgCl_2$, 10 mM ATP, 67 mM phosphate buffer, pH 7.0, as described earlier [14,29]. The resultant ghost fibers were composed of actin by more than 80%. S1 and TM were incorporated into pure F-actin filaments by incubation of the fibers in the solution containing 50 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 10 mM Tris–HCl, pH 6.8, and 1.0–2.5 mg/ml protein. The order of the incorporation of proteins into the ghost fibers was as follows: TM, S1. The unbound proteins were washed out by incubation of the fibers in the same buffer without proteins. FITC-phalloidin was tightly bound to F-actin of the fibres by their incubation in a solution containing 6.7 mM phosphate buffer (pH 7.0), 50 mM KCl, 3 mM $MgCl_2$ and 40 μM FITC-phalloidin for 2.5 h at room temperature [39].

The effectiveness of the reconstitution of filaments in ghost muscle fibres used for fluorescent measurements was verified by examining the protein content by SDS–PAGE with subsequent densitometry of the gels (UltroScan XL, Pharmacia LKB). The fibres in the final preparations contained actin, myosin subfragment-1, recombinant TM and Z-line proteins. The molar ratio of TM to actin was 1:6.5 (±2) irrespective of whether these proteins were modified by 5-IAF or not. In the absence of the nucleotides and in the presence of ADP, AMP-PNP, ATPγS, and ATP the molar ratio of S1 to actin was 1:5 (±2), 1:5 (±2), 1:8 (±2), 1:12 (±2), and 1:14 (±2), respectively.

Fluorescence polarization measurement

Steady-state fluorescence polarization measurements on single ghost muscle fibres were made using a flow-through chamber and photometer [39]. The polarized fluorescence from IAF-labelled TM and FITC-labelled actin was recorded at 500–600 nm after excitation at 489 ± 5 nm. Probes in ghost fibres were excited by a 250 W mercury lamp DRSH-250. The exciting light was passed through a quartz lens and a double monochromator, and split into