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The interchain disulfide cross-linking of tropomyosin alters its regulatory properties and interaction with actin filament

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ABSTRACT

Tropomyosin (Tpm) is an α-helical coiled-coil actin-binding protein that plays a key role in the Ca^{2+}-regulated contraction of striated muscles. Two chains of Tpm can be cross-linked by formation of a disulfide bond between Cys-190 residues. Normally, the SH-groups of these residues in cardiac muscle are in reduced state but in heart pathologies the interchain cross-linking of Tpm was shown to occur. Previous studies have shown that this cross-linking increases the thermal stability of the C-terminal part of the Tpm molecule. However it was unclear how this affects its functional properties. In the current work, we studied functional features of cross-linked Tpm at the level of isolated proteins. The results have shown that the cross-linking greatly decreases affinity of Tpm for F-actin and stability of the Tpm–F-actin complex. It also increases sliding velocity of regulated thin filaments in an *in vitro* motility assay. This last effect was mostly pronounced when cardiac isoforms of myosin and troponin were used instead of skeletal ones. The results indicate that cross-linking significantly affects properties of Tpm and actin-myosin interaction and can explain, at least partly, the role of the interchain disulfide cross-linking of cardiac Tpm in human heart diseases.

1. Introduction

Tropomyosin (Tpm) is an actin-binding, α-helical coiled-coil protein that binds along the length of an actin filament and plays, together with troponin (Tn) complex, a key role in Ca^{2+}-regulated contraction of striated muscles. According to recent views, Tpm acts as a steric blocker, cooperatively shielding and exposing myosin-binding sites on the surface of actin filaments [1–3]. In mammalian cells, up to 40 different Tpm isoforms are expressed from four different genes due to alternative splicing and alternative promoters. Among them, the product of human *TPM1* gene α-Tpm (or Tpm 1.1) is a predominant Tpm isoform in cardiac and fast skeletal muscles (onwards denoted as simply Tpm) [1]. Each chain of this Tpm isoform contains in its C-terminal part one cysteine residue at position 190. The two α-chains of the dimeric Tpm molecule are in register, and therefore they can be cross-linked by formation of a disulfide bond between the Cys-190 residues under oxidizing conditions [4]. Normally, the SH-groups of these residues are in a reduced state in skeletal or cardiac muscle [5,6]. However, the interchain cross-linking between Cys-190 residues of cardiac Tpm was shown to occur at myocardial dysfunction following coronary microembolization [7] and human end-stage heart failure [8]. On the basis of these data Canton and co-workers suggested that conformational changes induced by interchain cross-linking in the Tpm molecule may affect contractile function of cardiac muscle and in this way contribute to myocardial dysfunction [7,8]. To check this assumption, it is very important to know how this cross-linking affects structural and functional properties of Tpm.

About forty years ago Lehrer and co-workers have shown, using circular dichroism (CD) and differential scanning calorimetry (DSC), that the interchain disulfide cross-linking between Cys-190

Abbreviations: Tpm, tropomyosin; Tn, troponin.

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residues significantly increases the thermal stability of the Tpm molecule [9,10]. Following detailed DSC studies have shown that this cross-linking strongly stabilizes the C-terminal part of the Tpm molecule, with no appreciable effect on its N-terminal part [11–13]. Thus, structural properties of cross-linked Tpm have been intensively studied to date. Much less is known about the effects of this interchain cross-linking on the functional properties of Tpm.

There were only a couple of works published more than thirty years ago, which showed that cross-linked Tpm binds more weakly to actin filaments than uncross-linked one [14] and its inhibitory effect on the actomyosin ATPase in the absence of Tn is weaker than that of Tpm with the SH-groups of Cys-190 blocked with N-ethylmaleimide [15]. However, cross-linked Tpm and the N-ethylmaleimide-blocked Tpm were found to be indistinguishable in their effects on the actomyosin ATPase in the presence of Tn at any Ca^{2+} concentration [15]. These results seem to be rather doubtful as would imply that significant conformational changes induced by disulfide cross-linking in the Tpm molecule have no influence on the regulatory properties of Tpm and on the contractile function of cardiac muscle. On the whole, little is known so far about the functional properties of cross-linked Tpm and further studies are needed.

In the present work we focused on the effects of the interchain cross-linking of Tpm on its functional properties. For this we employed various methods, including the *in vitro* motility assay, to compare the properties of cross-linked Tpm with those of Tpm with SH-groups of Cys-190 in fully reduced state.

2. Materials and methods

2.1. Protein preparations

Tpm used in this work was recombinant human Tpm1.1 isoform (α-striated Tpm) with an Ala-Ser N-terminal extension to imitate naturally occurring acetylation of native Tpm [16]. As described earlier [17], Tpm was overexpressed in BL21(DE3) bacterial cells according to standard methods, extracted by heating, fractionated by reducing the pH to 4.8, and purified by anion exchange chromatography using a HiTrap QXL column.

To obtain Tpm in fully reduced state, it was heated at 60 °C for 45 min in the presence of 3 mM DTT. After such a procedure, all Tpm samples were in the fully reduced state [12,13]. The Tpm species cross-linked by disulfide bonds were obtained by the use of 5,5'-dithiobis(2-nitrobenzoate) (DTNB) as was earlier described by Lehrer [4]. Prior to DTNB treatment, SH-groups of Cys-190 were fully reduced as described above, and then the excess DTT was removed using NAP5 column (GE Healthcare). The content of cross-linked and reduced Tpm dimers was determined as described earlier [12], by SDS-PAGE [18] under non-reducing conditions, in the absence of β-mercaptoethanol or DTT.

Actin from rabbit skeletal muscle was prepared by the method of Pardee and Spudich [19], and monomeric actin was polymerized to actin filaments by the addition of 4 mM $MgCl_2$ and 100 mM KCl. Before experiments DTT was fully removed from F-actin by dialysis to prevent possible reduction of disulfide bonds in cross-linked Tpm species during the experiments. For experiments in the *in vitro* motility assay, F-actin was labeled by a 2-fold molar excess of TRITC-phalloidin (Sigma-Aldrich).

Myosin and Tn were prepared by established standard methods [20,21]. Myosin was extracted either from *m. psoas* of the rabbit or from the left ventricles of rabbit hearts (onwards denoted as skeletal or cardiac myosin, respectively); Tn was obtained either from rabbit *m. psoas* or from the left ventricles of bovine hearts (onwards denoted as skeletal Tn or cardiac Tn, respectively). Regulated thin filaments were reconstituted from F-actin, Tn, and Tpm according to Gordon et al. [22].

All procedures involving animal care and handling were performed according to institutional guidelines set forth by Animal Care and Use Committee at Institute of Immunology and Physiology Ural Branch of RAS and Directive 2010/63/EU of the European Parliament.

2.2. Cosedimentation and quantitative electrophoresis

The affinity of Tpm species for actin was estimated using a cosedimentation assay as described earlier [12,17]. F-actin (10 μM) stabilized by addition of a 1.5-fold molar excess of phalloidin (Sigma-Aldrich) was mixed with increasing concentrations of Tpm (0–3 μM) at room temperature in 30 mM Hepes, pH 7.3, 100 mM NaCl, to a final volume of 100 μl. After 40 min incubation, actin was pelleted with any bound Tpm by ultracentrifugation at 133,000 g for 40 min (Beckman airfuge, Beckman Instruments Inc., USA). Equivalent samples of the pellet and the supernatant were run on SDS–PAGE [18]. Quantification of protein bands was carried out by densitometry and scanned images were analyzed using the ImageJ 1.45s software (Scion Corp., Frederick, MD).

2.3. Light scattering

Thermally induced dissociation of Tpm–F-actin complexes was detected by changes in the light scattering at 90° as described earlier [12,17]. The measurements were performed at 350 nm on a Cary Eclipse fluorescence spectrophotometer (Varian Australia Pty Ltd, Mulgrave, Victoria, Australia) equipped with temperature controller and thermoprobes. All measurements were performed at a constant heating rate of 1 °C/min. Scattering of F-actin solutions containing the same concentration of actin (20 μM) as that in the Tpm–F-actin samples was measured before the experiments. Thermally induced Tpm dissociation from F-actin was accompanied by a decrease in the light scattering intensity, and therefore the temperature at which dissociation occurs can provide valuable information on the stability of the Tpm–F-actin complexes. The dissociation curves with temperature dependence of the light scattering for F-actin alone deducted were analyzed by fitting to a sigmoidal decay Boltzman function. The main parameter extracted from this analysis is T_{diss}, *i.e.* the temperature at which a 50% decrease in the light scattering occurs.

2.4. In vitro motility assay

Protocol of measurements of sliding velocity of regulated thin filaments at different Ca^{2+} concentrations in the *in vitro* motility assay was described earlier as well as composition of the buffers used [17]. In the experiments with cross-linked Tpm all buffers were DTT-free, the Ca^{2+}/EGTA motility buffer contained 5 mM 2-mercaptoethanol to avoid the reducing of SH-groups. It was checked in control experiments that a 30 min incubation with 5–10 mM 2-mercaptoethanol did not notably reduce SH-groups of Tpm. For the experiments with reduced Tpm, the buffers contained 10 mM DTT.

The experiments were done at 30 °C, sliding velocities of 30–100 filaments were measured using the GMimPro software [23]. Experiments were repeated three times with each of the Tpm species and the means of individual experiments were fitted to the Hill equation: $v = v_{max}(1 + 10^{n(pCa-pCa50)})^{-1}$, where v and v_{max} are velocity and maximal velocity at saturating calcium concentration, respectively, pCa_{50} (*i.e.* calcium sensitivity) is pCa at which half maximal velocity is achieved, and n is the Hill coefficient. All values are expressed as mean ± S.D. All comparisons were performed by Mann-Whitey U test ($p < 0.05$). The experiments with Tpm-actin filaments were performed under the same conditions, with the only exception that Tn and Ca^{2+} were not added.

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