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Flight muscle-specific Pro-Ala-rich extension of troponin is important for maintaining the insect-type myofilament lattice integrity

Hiroyuki Iwamoto *

Research and Utilization Div., SPring-8, Japan Synchrotron Radiation Research Institute, 1-1-1 Kouto, Sayo-cho, Sayo-gun, Hyogo 679-6198, Japan

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ABSTRACT

Insect flight muscle (IFM) can oscillate at frequencies up to 1000 Hz, owing to its capability of stretch activation (SA). It is a highly specialized form of cross striated muscles, and its peculiar features include the IFM-specific isoform of troponin-I (troponin-H or TnH) with an unusually long Pro-Ala-rich extension at the C-terminus. Although we have shown that this extension does not directly take part in SA, questions remain as to what its real role is and why it is expressed only in IFM. Here we explored the structural role of the extension, be comparing X-ray diffraction patterns and electron micrographs of bumblebee IFM fibers before and after enzymatic removal of the extension. The removal had a dramatic effect on diffraction patterns: In IFMs in general, the equatorial 2,0 reflection is much stronger than the 1,1 reflection, but after removal, their intensities became almost equal (stronger 1,1 is a feature of vertebrate skeletal muscle). Electron micrographs revealed that a substantial fraction of the thin filaments showed a tendency to move towards the vertebrate position (the trigonal position between three thick filaments), while the rest of the thin filaments remained in their original insect position (midway between two neighboring thick filaments). Therefore, one of the roles of the extension is suggested to keep the filament lattice in the correct configuration for IFM. This insect-type lattice structure is preserved among IFMs from varied insect orders but not in body muscles, suggesting that the maintenance of this lattice structure is important for flight functions.

1. Introduction

Small winged insects must beat their wings at high frequencies to obtain necessary lift (Dickinson, 2006), and the frequencies reach ~1000 Hz in some species (Sotavalta, 1953). These frequencies are beyond the range manageable by the repetition of contraction-relaxation cycles, caused by the release and re-uptake of intracellular calcium. Instead, these small insects have developed an asynchronous type of flight muscles, in which intracellular calcium is kept constant by low-frequency motor nerve impulses, while the two antagonistic flight muscles maintain autonomous oscillations by alternately pulling each other (Pringle, 1978; Josephson et al., 2000). These alternate pulls are driven by the mechanism of stretch activation (SA), i.e., delayed increase of active force induced by a stretch. SA is a conspicuous feature of the asynchronous variety of insect flight muscle (IFM), but is observed, although to a lesser extent, in other types of muscles as well, including vertebrate skeletal and cardiac muscles.

Asynchronous IFM is a highly specialized form of cross striated muscles, and many of its constituent proteins are IFM-specific isoforms. Among others, IFM troponin-I (troponin-H or TnH; Bullard et al., 1988) is the most peculiar, in that it has a long Pro-Ala-rich extension at the C-terminus, and its apparent molecular mass on the gel is 70–80 kDa (non-IFM isoforms, ~25 kDa). In our previous study we tested whether this unusual extension takes part in SA, but we found that its enzymatic removal from skinned IFM fiber specimen did not affect the capacity for SA (Iwamoto, 2013). This result raises new questions as to what function the extension performs, and why it is expressed only in IFM, if its role is not directly relevant to SA.

One suggestion for the role of the TnH extension comes from the observation that the homologous structure in *Drosophila* IFM (which is not associated with troponin-I but with tropomyosin) binds an isoform of glutathione-S-tranferase (Clayton et al., 1998), which is capable of processing oxidative stress caused by high mitochondrial activities (Singh et al., 2001; Agianian et al., 2003).

Here we explored the structural aspects of the role of the TnH extension, by using the techniques of X-ray diffraction and electron microscopy. As in the previous study, we applied an endoproteinase (Igase) to skinned bumblebee IFM fibers, and compared the X-ray diffraction patterns and the electron micrographs from the treated fibers with those from control. Igase has been shown to specifically and effectively remove the extension, while leaving the contractile properties largely intact (Iwamoto, 2013). An unexpected outcome

* Fax: +81 791 58 2512.
E-mail address: iwamoto@spring8.or.jp

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of this study is that, under relaxing conditions, the loss of the extension converts the myofilament lattice structure from the insect type to the vertebrate type. Although this structural transformation is reversed in activated fibers, the results suggest that the extension also has a structural role of maintaining lattice integrity. The insect-type lattice structure seems to be preserved among IFMs from all winged insect orders examined, suggesting that the maintenance of that lattice structure is important for IFM functions (not necessarily for SA). A brief account of a part of the present study has appeared (Iwamoto and Yagi, 2012).

2. Materials and methods

2.1. Muscle fiber specimens

The bumblebee (*Bombus* sp.) IFM fibers were prepared as described elsewhere (Iwamoto, 2009). Each single fiber was split to 2 strips before mounting for X-ray recordings. For X-ray recordings, seven of these strips were mounted on an experimental chamber at a time. The samples were 4–5 mm long. The specimen chamber was identical to the one used for previous studies (Tamura et al., 2009; Iwamoto et al., 2010).

2.2. Solutions

The compositions of the solutions were as described earlier (Iwamoto, 1995, 2000, 2009; Iwamoto et al., 2010). The relaxing, pre-activating and contracting solutions contained 20 mM imidazole, 5 mM $MgCl_2$ (free $[Mg^{2+}]$ = 0.75 mM), 4 mM Na_2ATP, 20 mM creatine phosphate and 125–700 U/ml creatine phosphokinase (pH = 7.2), ionic strength was adjusted to 193 mM by adding varying concentrations of K-propionate. In addition, the relaxing and activating solutions contained 10 mM EGTA, which was reduced to 0.1 mM in the pre-activating solution. The activating solution contained 10.4–10.1 mM of $CaCl_2$ to make pCa = 4.0–4.5. The rigor solution contained 120 mM K-propionate, 20 mM imidazole, and 5 mM each of EGTA and EDTA. Solutions for X-ray recordings contained 2 mM dithiothreitol and 1000 U/ml catalase.

2.3. Enzymatic removal of troponin-H extension

Enzymatic removal of TnH extension was performed as described (Iwamoto, 2013). Briefly, the skinned fibers were incubated overnight (~12 h) at 4 °C in a rigor solution containing endoproteinase Pro-Pro-Y-Pro (Igase, MoBiTec, Goettingen, Germany) (Clayton et al., 1998). After treatment, the fibers were washed thoroughly in the rigor solution. The overnight incubation has been shown to remove the extension from ~97% of troponin molecules (Iwamoto, 2013).

2.4. X-ray diffraction recordings

Static, conventional X-ray fiber diffraction patterns were recorded at the BL45XU beamline of SPring-8 (Fujisawa et al., 2000), by using a cooled CCD camera (C4880, Hamamatsu Photonics, Hamamatsu, Japan) in combination with an image intensifier (VP5445, Hamamatsu Photonics), and were analyzed as described previously (Iwamoto, 2009; Iwamoto et al., 2003, 2010). The patterns were recorded either in the relaxing solution or during isometric contraction. To activate the fibers, they were first placed in the pre-activating solution and then in an activating solution (Iwamoto, 2009). From each mounted array of muscle fibers, 20–26 diffraction patterns were recorded for each solution. After each exposure, the chamber was moved by 200 μm along the fiber axis to reduce radiation damage.

X-ray microdiffraction patterns from single myofibrils were recorded at the BL40XU beamline (Inoue et al., 2001). End-on diffraction patterns (Iwamoto et al., 2002, 2006, 2007) were recorded after rapidly freezing the myofibrils in the relaxing solution containing 20% methylpentanediol as a cryoprotectant. The mounting technique was not like in these studies but identical to the one used by Nishiura et al. (2012) to record end-on diffraction patterns from single flagellar axonemes: isolated single myofibrils were mounted straight right in the middle of a fine glass capillary before they were plunged into liquid propane.

2.5. Electron microscopy

Skinned relaxed fibers were fixed in the relaxed state by the mixture of 4% paraformaldehyde and 2% glutaraldehyde, and post-fixed with 2% osmium tetroxide. The fibers were then dehydrated and embedded in an epoxy resin (Quetol-812, Nisshin EM, Tokyo, Japan), thin-sectioned (30 nm) by using a ultramicrotome (ULTRACUT UCT, Leica Microsystems, Wetzlar, Germany), stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope (JEM-1200EX, JEOL, Akishima, Japan). The micrographs were recorded in a digitized form (2048 × 2048 pixels), and were further processed by using software built in house.

3. Results

3.1. X-ray diffraction patterns

We confirmed by electrophoresis that the TnH extension was effectively removed by the overnight Igase treatment (Iwamoto, 2013). Fig. 1 shows diffraction patterns from relaxed bumblebee IFM fibers, either control or fibers treated with Igase overnight. As has been published previously (Iwamoto, 2009; Iwamoto et al., 2010), the diffraction pattern from bumblebee IFM is typical of asynchronous IFMs, consisting of many layer-line and meridional reflections as well as a number of equatorial reflections. The lower-order layer line reflections are finely sampled by the filament lattice, because of the near-crystalline arrangement of sarcomeric proteins. Because of the match of the repeats of monomers in the thick and thin filaments, both myosin and actin contribute to the 1st and 2nd layer line reflections, but troponin has a major contribution to their innermost spots (the 1,0 row-line spots) (Tregear et al., 1998). Some of the lower-angle meridional reflections are also ascribed to troponin, because they are indexable to the troponin repeat (38.7 nm or 77.4 = 38.7 × 2 nm; Iwamoto et al., 2010).

Compared with the pattern from control fibers (Fig. 1A), the troponin reflections from Igase-treated fibers are generally weaker (Fig. 1B). In addition, a notable change is observed in the equatorial reflections: In IFM in general, the 2,0 reflection is much stronger than the 1,1 reflection, and unlike in vertebrate skeletal muscle, the relative intensities of the equatorial reflections hardly change upon activation. After Igase treatment, however, the 2,0 reflection becomes much weaker and 1,1 reflection stronger, and their intensities become almost equal. This tendency is also confirmed in the end-on diffraction patterns from the relaxed single myofibrils isolated from control and Igase-treated IFM fibers (Fig. 1C and D).

In the end-on pattern from the Igase-treated myofibril, the reflection spots are blurred, indicating that the hexagonal lattice of myofilaments is somewhat disordered. The disorder is also confirmed by the deterioration of lattice sampling in the first layer line reflection in the conventional diffraction pattern (Fig. 1B).

Fig. 1 also shows the magnified views of equatorial reflections from control (Fig. 1E) and Igase-treated (Fig. 1F) fibers, in the relaxed state and during activation. During activation of Igase-treated fibers, the normal intensity ratio of the 1,1 and 2,0 reflections