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A method for 3D-reconstruction of a muscle thick filament using the tilt series images of a single filament electron tomogram

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ABSTRACT

Myosin interacting-heads (MIH) motifs are visualized in 3D-reconstructions of thick filaments from striated muscle. These reconstructions are calculated by averaging methods using images from electron micrographs of grids prepared using numerous filament preparations. Here we propose an alternative method to calculate the 3D-reconstruction of a single thick filament using only a tilt series images recorded by electron tomography. Relaxed thick filaments, prepared from tarantula leg muscle homogenates, were negatively stained. Single-axis tilt series of single isolated thick filaments were obtained with the electron microscope at a low electron dose, and recorded on a CCD camera by electron tomography. An IHRSR 3D-recontruction was calculated from the tilt series images of a single thick filament. The reconstruction was enhanced by including in the search stage dual tilt image segments while only single tilt along the filament axis is usually used, as well as applying a band pass filter just before the back projection. The reconstruction from a single filament has a 40 Å resolution and clearly shows the presence of MIH motifs. In contrast, the electron tomogram 3D-reconstruction of the same thick filament – calculated without any image averaging and/or imposition of helical symmetry – only reveals MIH motifs infrequently. This is – to our knowledge – the first application of the IHRSR method to calculate a 3D reconstruction from tilt series images. This single filament IHRSR reconstruction method (SF-IHRSR) should provide a new tool to assess structural differences between well-ordered thick (or thin) filaments in a grid by recording separately their electron tomograms.

1. Introduction

Striated muscle consists of overlapping arrays of thick (myosin-containing) and thin (actin-containing) filaments. The contraction of muscle occurs when both sets of filaments slide relative to each other to produce sarcomere shortening (Huxley, 1969). This process is regulated by molecular switches located on either the thin filaments (troponin/tropomyosin) or the thick filaments, usually in the regulatory light chain of the myosin. To advance towards the understanding of the molecular mechanism of the myosin-linked regulation of muscle contraction, the determination of the structure of the myosin thick filaments is a requirement. The thick filaments form by tight packing of the long C-terminal, α-helical coiled-coils of the myosin heavy chains. The N-terminal domains of each heavy chain form a globular head, which contains the actin binding and ATPase activities. The tails pack together forming the backbone of the thick filament, and the myosin heads protrude from the backbone surface, often as an organized helical array (Craig and Padrón, 2004).

The structure of the thick filaments has been studied by X-ray diffraction (Huxley and Brown, 1967) which revealed the helical organization of the myosin heads on the surface of the thick filament. Initial structural studies by electron microscopy of negatively stained thick filaments (Huxley, 1963) revealed that the thick filaments were bipolar, with a central bare zone naked of myosin heads and disordered heads protruding on both sides. The improvement of the negative staining technique preserved helices of myosin heads in *Limulus* (Stewart et al., 1981), tarantula (Crowther et al., 1985) and scorpion (Kensler et al., 1985) thereby facilitating calculation of Fourier–Bessel 3D image reconstructions of the thick filaments (Stewart et al., 1981; Crowther et al., 1985; Kensler et al., 1985). However the resolution achieved (50 Å) was insufficient to resolve individual heads of each myosin molecule.

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Rapidly freezing thick filaments and observing them frozen-hydrated in the cryo-electron microscope at low electron dose can achieve higher resolution. However, due to problems separating overlapping Bessel functions when using Fourier–Bessel 3D-reconstruction procedures on such low contrast specimens (Padrón and Alamo, 2004), the improved specimen preservation could not be fully used to resolve both myosin heads. It was a different approach, the Iterative Helical Real Space Reconstruction (IHRSR) method (Egelman, 2000) that finally allowed individual myosin heads to be resolved in 3-D from electron micrographs of frozen-hydrated thick filaments (Woodhead et al., 2005). The two heads of each myosin molecule were found to assume an asymmetric structure we call the myosin interacting-heads (MIH) motif, similar to the one found in myosin 2D-crystals (Wendt et al., 1999, 2001; Liu et al., 2003). This motif has been detected by cryo-electron microscopy (EM) in *Limulus* and scallop (Zhao et al., 2009; Woodhead et al., 2013) and by negatively staining in mouse cardiac, scorpion and human cardiac (Zoghbi et al., 2008; Pinto et al., 2012; AL-Khayat et al., 2013) muscle thick filaments. A modification of the IHRSR method that takes into account the out-of-plane angle of the filaments (i.e. the angle that the filament made with the grid plane), increased the number of image segments that could be included in the reconstruction, and achieved a resolution of 20 Å (Alamo et al., 2008). The use of this modification together with band-pass filtering have facilitated detection of MIH motifs in scorpion thick filaments preserved using negative staining instead cryo-EM (Pinto et al., 2012).

This IHRSR 3D-reconstruction approach requires the recording of numerous electron micrographs from many different thick filament preparations imaged from several grids. Therefore the reconstructed MIH motifs are the average of potentially multiple different head conformations distributed on different thick filaments. Here we have recorded a tilt series of a single thick filament by electron tomography and enhanced the IHRSR method to calculate a 3D-reconstruction from it. This method successfully made possible to obtain a 3D-reconstruction from a single thick filament and validates the conclusion that the MIH motifs are the dominant structure on these relaxed thick filaments.

2. Materials and methods

2.1. Solutions

Relaxing solution contained 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 5 mM PIPES, 5 mM NaH_2PO_4, 1 mM NaN_3 and 5 mM Mg.ATP, pH 7.0. The permeabilizing solution consisted of relaxing solution containing 0.1 % (w/v) saponin.

2.2. Preparation of filament suspensions from tarantula striated muscle

Tarantulas (*Gramostola rosea*) were obtained from Carolina Biological Supply (Burlington, NC). Leg muscles were permeabilized for 3 h in relaxing-saponin solution and then washed for 1 h in relaxing solution. Saponin permeabilized muscle was homogenized for ~1 s in 3 ml of relaxing solution at setting of 5 on a Polytron homogenizer (Kinematica AG, Luzern, Switzerland). The homogenate was centrifuged at 15,000*g* (11,500 RPM) on a JA-20 rotor in an Eppendorf model 5415 D centrifuge (Eppendorf, Hauppauge, NY) for 2 min in a 4 °C cold room to remove large debris. The supernatant containing the thick filaments was stored and used the same day. The relaxed state of the thick filaments as judged by their helical order was not easily obtained. The best pH for getting relaxed state was 6.8. Most of the negative stained preparations were disordered.

2.3. Electron tomography

A thick filament suspension was applied to glow discharged holey carbon grids with a thin carbon film suspended over the holes and negatively stained with 2% uranyl acetate. Conventional single-axis tilt tomographic series (total accumulated electron dose ~473 e^-/A^2 for 66 tilt images) were obtained with a FEI CM-120 electron microscope (FEI Company, Eindhoven, The Netherlands) using a 3° Saxton scheme (Saxton et al., 1984) from +69.6° to −69.3° and recorded on a 2 K TVIPS CCD camera (Tietz Video and Image Processing Systems GmbH, Gauting, Germany) at a 8.5 μm defocus. The step size starts at 0.3° at the high positive tilt angles and 1.1° for the negative ones. The pixel size with respect to the original specimen is 0.668 nm. The tilt series was aligned using marker-free alignment and tomograms calculated by weighted back projection using the Protomo software package (Winkler and Taylor, 2006; Winkler, 2007).

2.4. Single filament IHRSR (SF-IHRSR) 3D reconstruction method and validation

We have enhanced the IHRSR method (Egelman, 2000) to fully reconstruct a single myosin thick filament from images within a tilt series. This "single filament IHRSR" 3D reconstruction method ("SF-IHRSR" method, Fig. 2) consists of: (1) collecting a single tilt series of images from a half of one single thick filament using electron tomography instead of collecting many electron micrographs from different thick filaments from many grids; (2) calculating an IHRSR 3D-recontruction from this tilt series of images by including in the search stage additional image segments with out-of-plane angles which are not normally included (Alamo et al., 2008), as well as applying a band pass filter just before the back projection (Pinto et al., 2012). The SF-IHRSR method was implemented in a SPIDER environment (Frank et al., 1996) and the authors can provide the SPIDER script if required. The IHRSR 3D-reconstruction shown in Fig. 4a was computed using only a subset of 21 images from −28.9° to +28.9° (Supplementary movie 1) of a well preserved region of one half of the filament. The 0° image is shown in Fig. 1a. The minimum filament tilt images required to obtain a reasonable reconstruction is 11 (Fig. 3a) corresponding to a ~30° tilt, this covers 0–90° rotational angles required by 4-fold symmetry in 3 crowns. For the SF-IHRSR 3D-reconstruction an arbitrary initial reference was used (Yang et al., 2003; Egelman, 2007). Supplementary Fig. 1c shows that the number of segments required for a stable IHRSR reconstruction was achieved in less than 10 cycles. After 40 cycles, 1013 segments from a total of 1673 were enough to obtain a resolution of 40 Å. In Fig. 3 it is shown the effect of reducing the number of images from the tilt series included in the SF-IHRSR 3D-reconstruction. It is seen that to obtain an informative 3D-reconstruction requires at least 5 tilt images (Fig. 3a). In contrast, the IHRSR 3D-reconstruction from one image of a single thick filament tilt series was very noisy (Fig. 3a). The Fourier Shell Correlation (FSC) plots of these IHRSR 3D-maps (Fig. 3b) show the increasing resolution achieved with the number of tilt images included in the 3D-reconstruction. To calculate the FSCs we used the SPIDER operator "BP 32F" that randomly splits the data sets from 21 tilt series images in two halves and calculate the corresponding volume map, then used the operator "FSC" to calculate the plot. Even though these two data sets were not completely independent (van Heel, 1987; Chen et al., 2013; Penczek, 2010) the low res map (40 Å) agrees with previous reconstructions using independent data sets (Pinto et al., 2012).

In Fig. 4 we compare this SF-IHRSR 3D-reconstruction calculated for one filament (Fig. 4a) with a multiple filament IHRSR 3D-reconstruction obtained either from negative stained (Fig. 4b, (Pinto et al., 2012)) or frozen-hydrated (Fig. 4c, (Alamo et al.,