J. Biochem. 123, 614-618 (1998)

Direct Observation of a Central Bare Zone in a Native Thick Filament Isolated from the Anterior Byssus Retractor Muscle of *Mytilus edulis* Using Fluorescent ATP Analogue

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Received for publication, October 2, 1997

To investigate the existence of a central bare zone in native thick filaments isolated from the anterior byssus retractor muscle (ABRM) of blue mussels (*Mytilus edulis*), the filaments were observed by fluorescence and dark-field microscopy after being incubated in the presence of Ca^{2+} with the fluorescent ATP analogue, Cy3-EDA-ATP. Filaments appeared under dark-field illumination as thin rods with tapered ends of length 5-30 μm. Fluorescence microscopy revealed that Cy3-EDA-ATP was bound to these filaments, except near their center. Although the boundary between this central non-fluorescent zone and fluorescent regions was not clearly defined, there was a trend for the width of the central non-fluorescent zone to increase with thick filament length (correlation coefficient=0.45; n=142). When Cy3-EDA-nucleotides bound to thick filaments were displaced by excess ATP, fluorescent images disappeared with a rate constant of 0.024 s^{-1}, close to the turnover rate of Cy3-EDA-ATP by myosin on the native thick filaments. These results indicate that each native thick filament isolated from the ABRM has a central bare zone, but its boundary was not sharply resolved.

Key words: central bare zone, Cy3-EDA-ATP, fluorescent ATP analogue, myosin, thick filament.

Muscle contraction is produced *via* the interaction of thick filaments, composed mainly of myosin molecules, which self-associate to form these filaments, and thin filaments composed principally of actin, which together hydrolyze ATP efficiently, slide past each other and so generate movement and force. Electron microscope observations of myosin filaments isolated from vertebrate skeletal muscles indicate that myosin molecules form bipolar filaments with a central bare zone (*1*). The presence of a central bare zone demonstrates that myosin filaments are bipolar. Although the structure of myosin filaments has been much studied, the details have still to be elucidated and vary in different species (*2*). Thus, in contrast to vertebrate striated muscle myosin filaments, the ultra-structure of smooth muscle myosin filaments is still a subject of interest. Indeed, the thick filaments of molluscan smooth muscle have a large paramyosin core covered by a surface layer of myosin molecules (*3-6*) and the molecular packing of myosin molecules on the paramyosin core remains unclear (*7*).

The structure of these thick filaments of molluscan smooth muscle has been studied by X-ray diffraction and electron microscopy (*8-10*). The X-ray diffraction studies showed the existence of helical repeats of myosin and paramyosin molecules in the thick filaments. On the other hand, electron microscopy revealed the surface structure of the thick filaments. However, X-ray studies provide only information about periodic structures and electron microscopy requires fixation and staining of the sample, which might give rise to artefacts. For these technical reasons, the structure of the central bare zone has not been extensively studied. In addition, the great length of the thick filaments has hampered the location of the bipolar region in electron microscopy.

The ribose-modified fluorescent ATP analogue, 2'(3')-*O*-[*N*-[2-[(Cy3)amido]ethyl]carbamoyl]-ATP (Cy3-EDA-ATP) is a good substrate for myosin and actomyosin ATPase with similar kinetic constants to those of native ATP (*11, 12*). Since the concentration of myosin heads on myosin filaments was higher than that of the free fluorescent ATP analogue in solution, the filaments were observed as fluorescent images under a fluorescence microscope when the filaments were incubated with sub-micromolar concentrations of fluorescent ATP analogues (*13, 14*). In addition, Cy3-EDA-ATP bound to myosin was completely displaced by excess ATP and little non-specific binding remained on myosin filaments (*14*).

In the present study, we have observed the central bare zone of native thick filaments isolated from molluscan smooth muscle, the anterior byssus retractor muscle (ABRM), using Cy3-EDA-ATP and revealed that the boundary of the central bare zone is not sharp, but rather that the number of myosin heads decreases gradually toward the center.

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MATERIALS AND METHODS

Preparation of Thick Filaments from the Anterior Byssus Retractor Muscle (ABRM) of Mytilus edulis—Blue mussels, *Mytilus edulis*, were obtained from a bay near Himeji. Native thick filaments of the ABRM were prepared basically according to the method of Yamada *et al.* (*15*). Two ABRMs were dissected from each animal and were split into thin strips. The strips were then washed and homogenized in chilled "filament buffer" containing 8 mM Na_2ATP, 10 mM $MgCl_2$, 2 mM EGTA, 2 mM dithiothreitol (DTT), and 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES)-NaOH (pH 7.0). The homogenate was centrifuged at 2,000 rpm for 5 min in a Kubota RA-53G rotor and the supernatant was collected. Triton X-100 was then added at 0.05% ($w \cdot v^{-1}$) to the supernatant and the filament suspension was incubated on ice for 30 min. The resultant solution was centrifuged at 8,000 rpm for 30 min and the precipitate obtained was subsequently re-suspended in filament buffer. This suspension was centrifuged at 2,000 rpm for 5 min and the supernatant was used for the experiments described.

Measurement of Cy3-EDA-ATPase of Native Thick Filaments of the ABRM—Cy3-EDA-ATP was synthesized according to the methods described by Jameson and Eccleston (*16*). Substituting the 2′- or 3′-hydroxyl group of the ribose moiety with probes generally results in a mixture of compounds. Thus, the Cy3-EDA-ATP we used in this study is an equilibrium mixture of 2′- and 3′-isomers (*16*). It was tested as substrate for *M. edulis* myosin ATPase as follows. Native thick filaments isolated from the ABRM (0.13 $mg \cdot ml^{-1}$) were incubated in a solution containing 25 mM KCl, 3 mM $MgCl_2$, 2 mM EGTA, 2.2 mM $CaCl_2$, 10 mM DTT, and 10 mM PIPES-KOH (pH 7.0), with 7.2 μM Cy3-EDA-ATP at 20°C. Aliquots were taken every minute, mixed with 10% perchloric acid and then centrifuged to remove proteins. The supernatant was loaded on a reverse phase column (Nova Pak C_{18}, 150 × 3.9 mm, Waters, MA) and eluted isocratically with 10% acetonitrile and 10 mM potassium phosphate buffer, pH 6.8, while monitoring the Cy3 fluorescence (excitation 545 nm, emission 565 nm). This procedure enabled us to separate Cy3-EDA-ATP from Cy3-EDA-ADP and provided a mean to measure Cy3-EDA-ATPase. Two closely spaced peaks were seen corresponding to elution of the 2′- and 3′-isomers in diphosphate form.

Observation of Myosin Filaments by Dark-Field Microscopy and Fluorescence Microscopy—A flow cell was prepared with two coverslips spaced by two slivers of 50-μm-thick polycarbonate film, basically according to Kron and Spudich (*17*). The flow cell was placed on the stage of an epi-fluorescence microscope (Diaphoto-TMD, Nikon, Tokyo) equipped with a dark-field condenser (DC-W, N.A. = 1.2-1.4, Olympus, Tokyo). This was placed on the opposite side of the flow cell from the objective, which enabled us to observe thick filaments as dark-field and fluorescent images simultaneously. For dark-field illumination, a 100 W halogen lamp was used. For fluorescence observations, a 100 W mercury lamp, with a band pass filter (EX510-560, Nikon) was used for excitation of Cy3-EDA-ATP. In order to separate excitation from emission light a dichroic mirror (DM580, Nikon) and a band pass filter (BA590, Nikon) selected for isolation of fluorescence from Cy3-EDA-ATP were used.

Isolated thick filaments (0.13 $mg \cdot ml^{-1}$) in a solution containing 25 mM KCl, 10 mM PIPES-KOH (pH 7.0), 3 mM $MgCl_2$, 2 mM EGTA, 2.2 mM $CaCl_2$, 10 mM DTT, 2 $mg \cdot ml^{-1}$ bovine serum albumin, 1 mM ATP, and oxygen depletion cocktail (0.02 $mg \cdot ml^{-1}$ catalase, 0.1 $mg \cdot ml^{-1}$ glucose oxidase, and 5 $mg \cdot ml^{-1}$ glucose) were introduced into the flow cell. Thick filaments were spontaneously attached sparsely to the surface of the coverslip. These myosin filaments were washed twice with the above solution, but without ATP, and then perfused with the above solution containing 100 nM Cy3-EDA-ATP instead of 1 mM ATP. The Cy3-EDA-nucleotides (triphosphate form and diphosphate form) bound to myosin are subject to photobleaching, which converts them to non-fluorescent forms. Perfusion of the flow cell with Cy3-EDA-ATP displaces photobleached Cy3-EDA-nucleotides with fresh free Cy3-EDA-ATP from the solution which has not been subject to significant photobleaching. Thus, the intensities of the fluorescent images of filaments were not diminished after illumination and the images could be observed for more than 10 min.

Both fluorescence and dark-field images of these filaments were collected *via* an oil-immersion objective lens (CF Achromat, 100×, N.A. = 0.9-1.25, Nikon) and projected onto an SIT camera (C2400-08, Hamamatsu Photonics, Hamamatsu). Video signals were processed with rolling-averaging, background subtraction, and contrast enhancement using an analogue image processor (Argus-10, Hamamatsu Photonics). These video signals were stored on S-VHS tapes (30 $frames \cdot s^{-1}$) and analyzed off-line using a frame-grabber to transfer data to a Macintosh computer running the Image Analyst software package (Automatix, Middlesex, MA).

RESULTS

Cy3-EDA-ATPase of Native Thick Filaments of ABRM—To test Cy3-EDA-ATP as a substrate for myosin obtained from ABRM, we measured the steady-state Cy3-EDA-ATPase in the presence of Ca^{2+}. Under the conditions we used, 7.2 μM Cy3-EDA-ATP was hydrolyzed steadily at a rate of 0.54 $nmol \cdot min^{-1} \cdot mg^{-1}$ thick filaments. This steady-state rate of Cy3-EDA-ATPase was comparable to the native maximum ATPase rate, 3.5 ± 1.6 $nmol \cdot min^{-1} \cdot mg^{-1}$ thick filaments obtained under similar conditions, but with 1-2 mM ATP (*15*), assuming a Michaelis-Menten K_m for ATP of 40 μM. For a molecular ratio of paramyosin to myosin of 2.88 : 1 (*6*), we calculated the turnover rate of myosin Cy3-EDA-ATPase to be 0.007 s^{-1}, with a V_{max} of *ca.* 0.05 s^{-1}. This result indicates that Cy3-EDA-ATP is as a good substrate for ATPase of molluscan smooth muscle myosin as for rabbit skeletal muscle myosin (*11*). These steady-state Cy3-EDA-ATPase measurements also showed that the amounts of 2′- and 3′-isomers in the diphosphate forms increased in the same proportion, consistent with the progress in hydrolysis of Cy3-EDA-ATP. Thus, it is likely that both the 2′- and 3′-isomers were hydrolyzed at equal rates by myosin.

Observation of the Central Bare Zone of a Native Thick Filament—Native thick filaments isolated from the ABRM were introduced into the flow cell and spontaneously

attached to its surface. The use of freshly prepared thick filaments minimized the formation of bundles of filaments, so that isolated thick filaments were sparsely attached to the surface. They were observed under a dark-field microscope as long spindle-shaped images with tapered ends (Fig. 1A). The length of the filaments ranged from 5 to 30 μm (16.1$\pm$4.6 μm, mean$\pm$standard deviation, n=142). Those filaments which formed bundles or were broken during preparation could be distinguished from single intact filaments under the dark-field illumination and they were excluded from the measurements.

Native thick filaments attached to the glass surface were first incubated with filament buffer containing 2.2 mM Ca^{2+}, 2 mM EGTA, and no ATP, and then buffer containing 100 nM Cy3-EDA-ATP was added to the flow cell. Assuming that K_m of Cy3-EDA-ATPase of myosin on these thick filaments is the same as that for rabbit skeletal muscle myosin (50 nM) (*14*), more than half of the myosin heads on a thick filament would have Cy3-EDA-ATP in their active sites at the concentration of 100 nM Cy3-EDA-ATP. Since the local concentration of myosin heads on the filaments is much higher than that of the free Cy3-EDA-ATP in solution, myosin heads with Cy3-EDA-ATP bound were detected as distinct fluorescent images (Fig. 1B). After examining the filaments as spindle-shaped images in the dark-field image, we observed the same filaments under epi-fluorescence. The gallery of dark-field images and fluorescent images of thick filaments in Fig. 2 shows that fluorescence is absent in the central zone of these filaments while long spindle-shaped continuous images with tapered ends were observed under the dark-field illumination. The intensity profile of fluorescence measured along a thick filament shows that the intensity did not change abruptly at the boundary but gradually decreased toward the center of the filament (Fig. 3), in spite of the fact that each thick filament is actually widest in the center (*15*). This observation suggests that the number of myosin molecules on a thick filament gradually decreases toward the center.

The width of the non-fluorescent zone at the center of a filament was defined as the width of that region where the fluorescence was less than 10% of the maximum fluorescence observed on the same filament. The relationship between the length of the filaments and the width of the non-fluorescent zone is shown in Fig. 4. Although the distribution is scattered, it indicates that longer filaments tend to have wider non-fluorescent zones with a correlation coefficient of 0.45 (n=142).

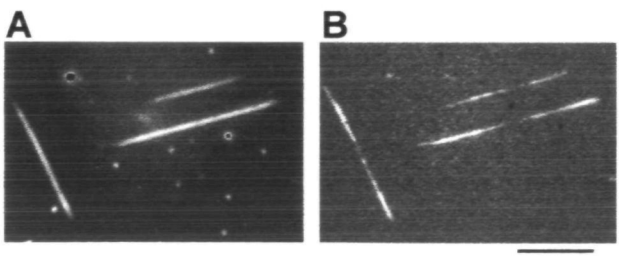

Fig. 1. **Images of native thick filaments of the ABRM obtained from dark-field microscopy (A) and fluorescence microscopy (B) in the presence of 100 nM Cy3-EDA-ATP.** Both panels show the same field of a flow cell. Thick filaments were easily identified as thin rods with tapered ends under dark-field microscopy. The scale bar represents 10 μm.

Displacement of Cy3-EDA-ATP from Immobilized Native Thick Filaments by Excess ATP—The native thick filaments were so firmly attached to the surface of the coverslip that they were not flushed away with perfusion of fresh buffer into the flow cell. Therefore, it was possible to

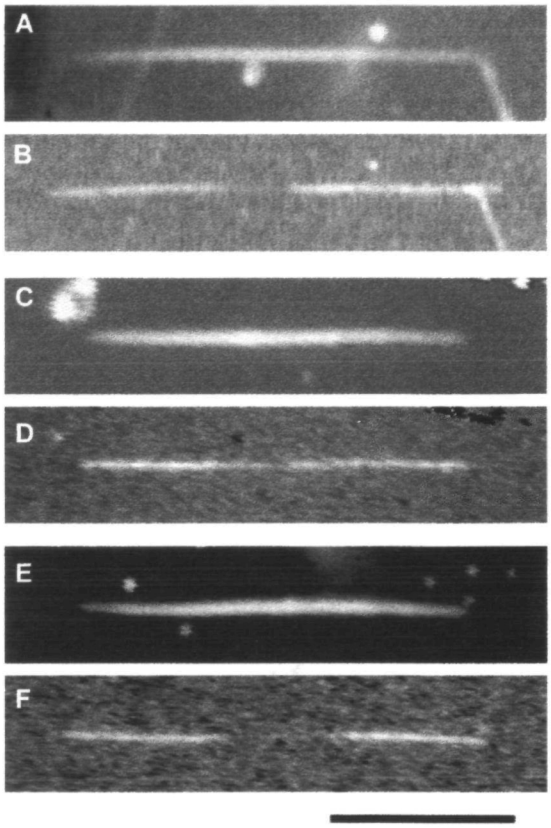

Fig. 2. **Gallery of fluorescence images and dark-field images of native thick filaments.** Panels (A), (C), and (E) were obtained under dark-field illumination. Panels (B), (D), and (F) were obtained by fluorescence microscopy. Note the central zones of the filaments, lacking fluorescence. The scale bar represents 10 μm.

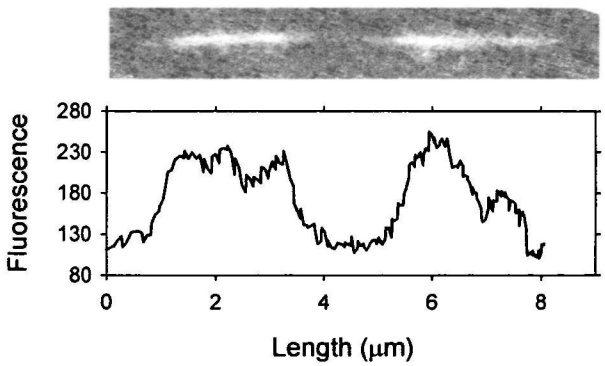

Fig. 3. **Fluorescence image of a native thick filament and the intensity profile of fluorescence measured along that filament.** Since the filament tapers towards the ends the fluorescence intensity decreases at both ends. In contrast, the intensity gradually decreases near the center of the filament despite the filament being widest in the center. The fluorescence image was processed with four-frame rolling averaging, but without background subtraction. The units of fluorescence intensity are arbitrary.

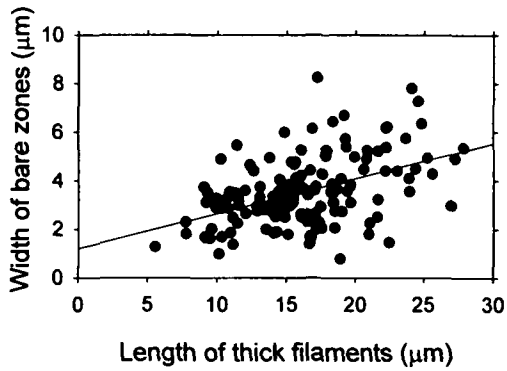

Fig. 4. **The relationship between the length of thick filaments and the width of their central bare zone.** The lengths of thick filaments were measured using the images recorded under dark-field microscopy. The width of the bare zone was obtained by using fluorescence microscopy on the same filaments as were observed in dark-field microscopy. The correlation coefficient of this relationship is 0.45 ($n=142$). The straight line represents a linear least-squares regression line (slope=0.14).

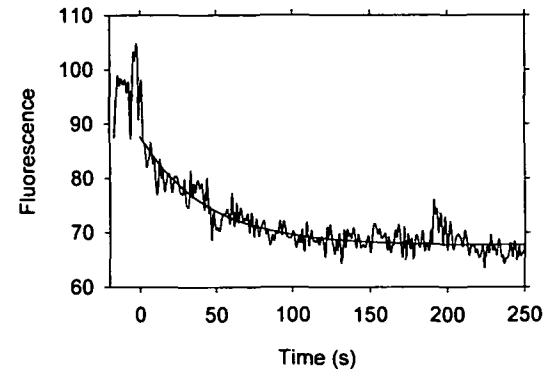

Fig. 5. **The decay in fluorescence intensity on myosin filaments elicited by perfusion of excess ATP.** The myosin filament was first incubated with 100 nM Cy3-EDA-ATP and then perfused with excess ATP (1 mM) in addition to Cy3-EDA-ATP. The fluorescence intensity became noisy around the time zero owing to the perfusion artefact. The fit of a single exponential to the decay of the fluorescence has a rate constant of 0.023 s^{-1}, close to the Cy3-EDA-ATPase rate (0.01 s^{-1}) obtained in a solution study.

record fluorescent signals at full video rate from the same immobilized thick filaments during the displacement of Cy3-EDA-ATP (-ADP) by ATP. When perfused with an excess of ATP in addition to Cy3-EDA-ATP, the fluorescent image of the filaments disappeared within 5 min (Fig. 5) and no fluorescence remained on the filament, since the reaction products of Cy3-EDA-ATP were displaced by ATP. The decay in fluorescence intensity of individual immobilized thick filaments could be fitted by a single exponential, the average rate of which was 0.024 ± 0.009 s^{-1} (mean$\pm$standard deviation, $n=15$). This value was in good agreement with that obtained for the turnover rate of Cy3-EDA-ATP in solution. In addition, the fact that no fluorescence remained on the filaments indicates that there was no significant amount of non-specific binding of Cy3-EDA-ATP to the thick filaments.

The rate of photo-decomposition was examined in the presence of 100 nM Cy3-EDA-ATP and was less than 0.002 s^{-1}. In comparison with the rate of ATP turnover in solution (0.01-0.04 s^{-1}), the rate of photo-decomposition was so slow that the effect of photo-decomposition of Cy3-EDA-ATP is negligible and the rate determined by displacing Cy3-EDA-ATP by excess ATP may be regarded as its turnover rate.

These results indicate that fluorescence of thick filaments in the presence of Cy3-EDA-ATP arises only from Cy3-EDA-ATP (or ADP) bound to the active sites of myosin and that there were few myosin molecules in the non-fluorescent zone in the central part of a filament.

DISCUSSION

In the present study, the existence of a central bare zone in native thick filaments of the ABRM of *Mytilus edulis* was clearly demonstrated and the quantitative relationship between the width of this bare zone and filament length was first determined. Based on the fluorescence intensity of myosin filaments, the boundary of the bare zone was not sharply defined but the number of myosin heads seemed to decrease gradually toward the center of the filament. In electron microscope observations of thick filaments obtained from the pedal retractor muscle (*9, 10*) and the ABRM (*15*), the bare zones were reported to be less prominent than those of vertebrate skeletal muscle myosin filaments. In the *in vitro* motility assay actin filaments move on the thick filaments of the ABRM. However, no movement of actin filaments was observed for *ca.* 3 μm of the central zone of a thick filament (*18*). Our results clearly confirmed these findings.

The mechanism by which vertebrate skeletal muscle myosin filaments form the bare zone in their center was first proposed by Huxley (*1*) and according to his scheme, the width of the bare zone is about the same length as the myosin tail (*ca.* 150 nm). This scheme would not be applicable to the thick filament of molluscan smooth muscle since myosin molecules cover only the surface layer of the paramyosin core (*3*) and the width of bare zone we measured (*ca.* 3 μm) was much larger than that of a myosin rod. Indeed, synthetic myosin filaments of rabbit skeletal muscle were observed as uniformly bright fluorescent images in the presence of 0.2-5 nM Cy3-EDA-ATP (*14*). No central bare zone of these synthetic myosin filaments could be observed. This is due to the narrow width (*ca.* 150 nm) compared to the low spatial resolution of the fluorescence microscope (*ca.* 600 nm).

It is well-known that native paramyosin filaments isolated from molluscan smooth muscle show a Bear-Selby net pattern (*19, 20*) and near their centers, the polarity of the net pattern is reversed. Owing to insufficient spatial resolution of fluorescence microscopy, it is difficult to discuss the structural details of the boundaries. However, the fact that the fluorescence intensity gradually decreased at the boundary indicates that the number of myosin molecules gradually decreased toward the filament center. This observation implies that the disorder of the paramyosin core might affect the structure of the myosin layer.

The ABRM is a "catch" muscle, which maintains high tension with little expenditure of energy. Acetylcholine induces catch contraction in the ABRM, whereas 5-hydroxytryptamine (5-HT) interrupts the catch and relaxes the muscle (*21*). The effect of 5-HT is mediated by cAMP which

may promote phosphorylation of paramyosin (*22*) and myosin (*23*). Though the mechanism of "catch" remains unknown, one hypothesis (*24*) is that there is an intimate relationship between myosin and paramyosin that can be changed under certain conditions. In this case, detachment of myosin from actin filaments could be hindered by the specific interaction between myosin and paramyosin. Phosphorylation of paramyosin and myosin could be involved in the regulation of the "catch" mechanism (*22*). Though the paramyosin core of the thick filament is almost covered with myosin at both ends of the filaments, the paramyosin core in the central bare zone is exposed directly to cytoplasm and so is accessible to phosphorylation. Hence, the central bare zone of the thick filament might be involved in the "catch" mechanism.

The present study also demonstrates that the fluorescent ATP analogue, Cy3-EDA-ATP is an excellent tool for observing small numbers of myosin molecules, since this analogue is a good substrate for myosin ATPase of native thick filaments and shows little nonspecific binding to those filaments. Due to the large extinction coefficient of Cy3-EDA-ATP and its resistance to photo-decomposition, Cy3-EDA-ATP has been used for kinetic measurements with single molecules (*14, 25*). Since Cy3-EDA-ATP has been proved here to be a good substrate for molluscan myosin, single-molecule kinetic techniques should also be applicable to molluscan thick filaments.

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