

The background of the cover is a complex, abstract network of glowing blue and white lines, resembling a microscopic structure or a neural network. The lines are interconnected and vary in thickness, creating a dense, web-like pattern. The overall color palette is dominated by deep blues and bright whites, giving it a high-tech, scientific feel.

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Dynamic electron microscopy: Novel properties of ATP-induced myosin head movement in living muscle myosin filaments

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Abstract

Muscle contraction is produced by relative sliding between actin and myosin filaments, which in turn is caused by cyclic attachment and detachment between myosin heads extending from myosin filaments and corresponding myosin-binding sites in actin filaments. When attached to actin filaments, myosin heads are believed to perform power stroke to produce unitary filament sliding, and then detach from them to perform recovery stroke to return to their initial position. Despite extensive studies, however, the myosin head movement still remains to be a matter for speculation, because it occurs asynchronously in contracting muscle.

Using the gas Environmental Chamber (EC), which enables us to record structural changes of wet, living biomolecules electron microscopically, we have succeeded in obtaining the following novel results on the ATP-induced myosin head movement in the absence of actin filaments: (1) Without ATP application, the position of myosin heads remains unchanged with time, indicating stability in myosin head time-averaged mean position; (2) On iontophoretic ATP application, myosin heads move away from, but not towards, the bare region at the middle of myosin filaments; (3) The average amplitude of myosin head movement was ~ 6 nm; (4) After exhaustion of applied ATP, myosin heads return to their initial position. These results indicate that myosin heads can determine the direction of their ATP-induced movement without being guided by actin filaments. We also obtained interesting results on myosin head power stroke in the actin-myosin filament mixture, and demonstrated that myosin head power stroke exhibits two different modes depending on the ionic strength of experimental solution. We emphasize that the EC is a powerful tool in investigating mysteries in muscle contraction.

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Introduction

A myosin molecule consists of two parts; (1) a rod of 113nm long, called light meromyosin (LMM), and (2) the rest of myosin molecule with two pear-shaped heads (S-1) and a rod of 43nm long (S-2), called heavy meromyosin (HMM) (Figure 1A). In myosin (or thick) filaments, LMM aggregates to form filament backbone, which is polarized in opposite directions on either side of the central part, called bare zone. On the other hand, myosin heads extend laterally from myosin filament backbone with axial interval of 14.3nm (Figure 1B). Actin (or thin) filaments con-

sist of two helical strands of globular actin monomer (diameter, 5.46nm) with a pitch of 35.5nm. They also contain two proteins, tropomyosin and troponin, which are concerned with regulation of actin-myosin interaction (Figure 1C). In muscle, actin and myosin filaments are arranged regularly with a structural and functional unit, called sarcomere. In each sarcomere, actin filaments extend in either direction from Z-line to penetrate in between myosin filaments, located centrally in each sarcomere (Figure 1D).



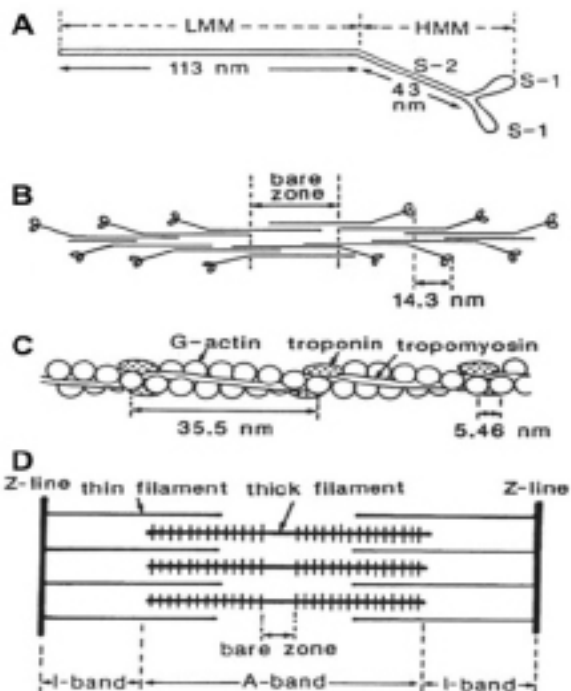


Figure 1: (A) Structure of myosin molecule. (B) Symmetrical myosin filament structure across the central bare region. (C) Structure of actin filament. (D) Symmetrical arrangement of actin (thin) and myosin (thick) filaments in a sarcomere [1].

It is generally believed that, in contracting muscle, myosin heads (M) first bind with actin filament (A) in the form of M-ADP-Pi, and perform power stroke to produce unitary filament sliding, with reaction, $A-M-ADP-Pi \rightarrow A-M + ADP$, and then detaches from (A) by binding next ATP to perform recovery stroke associated with reaction, $M-ATP \rightarrow M-ADP-Pi$, to restore their initial position (Figure 2). Consequently, power and recovery strokes are the same in amplitude and opposite in direction. The above myosin head movement is, however, still a matter for speculation. Despite extensive studies using time-resolved X-ray diffraction and chemical probes attached to myosin heads, no clear evidence for myosin head movement has been obtained, because myosin head movement takes place asynchronously in contracting muscle [2,3].

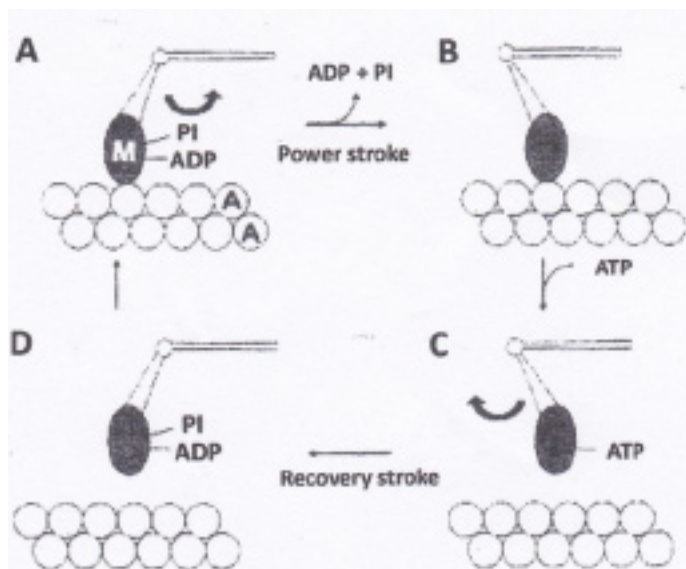


Figure 2: Diagram showing myosin head power and recovery strokes, associated with ATP hydrolysis. For explanations, see text [4].

The most straightforward way to obtain information about the myosin head movement, coupled with ATP hydrolysis, is to observe muscle myosin filaments in wet, living state under an electron microscope, and to record myosin head movement in response to ATP application under high magnifications. In the early 1980s, we met Professor Akira Fukami in Nihon University, who had just succeeded in constructing the gas environmental chamber (EC, or hydration chamber) attached to a transmission microscope [5], and agreed with him to work together. Here, we describe our methods to record ATP-induced myosin head movement together with the results on the novel properties of myosin heads in living myosin filaments.

Materials and methods

The gas environmental chamber (EC)

As illustrated in Figure 3, the EC is a metal compartment with upper and windows to pass electron beam. Each window is covered with a thin carbon insulating film, which bears pressure difference up to 1 atmosphere. The specimen, placed on the lower carbon film, is kept wet, living state by constantly circulating water vapor through the EC. The EC contained an ATP-containing glass microelectrode to apply ATP to the specimen by passing negative current to the electrode, so that negatively charged ATP molecules were released out of the electrode to reach the specimen by diffusion. The EC is attached to a transmission electron microscope (JEM 2000EX, JEOL). Further details have been described elsewhere [2,6].

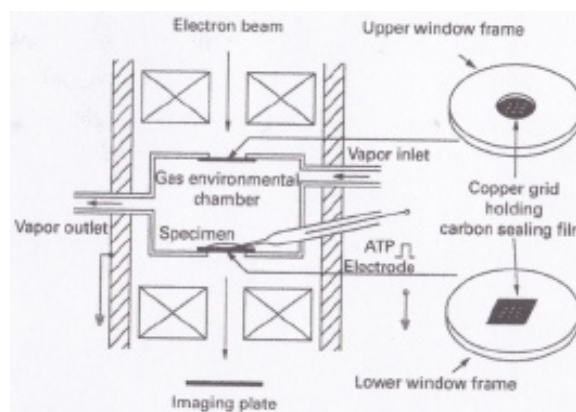


Figure 3: Diagram of the EC. The upper and lower windows to pass electron beam are covered with carbon sealing film. ATP is applied to the specimen by passing current to the ATP-containing electrode [7].

Preparation of Myosin Filaments

For technical reasons, we used spindle-shaped synthetic myosin filaments, having symmetrical myosin molecule arrangement across the middle of the filament, i.e. the bare region (see, Figure 1B). During muscle contraction, myosin heads attached to actin filaments perform power strokes to draw actin filaments into myosin filament arrays, the direction of their power stroke is towards the bare region, while that of recovery stroke is away from the bare region.

As the filaments are in living, unstained condition, it was necessary to position-mark individual myosin heads. We could successfully position-mark myosin heads by attaching them colloidal gold particles (diameter, 20nm) via three different antibodies; antibody 1 to the distal part of myosin head catalytic domain (CAD)[7], antibody 2 to the proximal region of myosin head CAD[7], and antibody 3 to myosin head lever arm domain

[8], as shown in Figure 4.

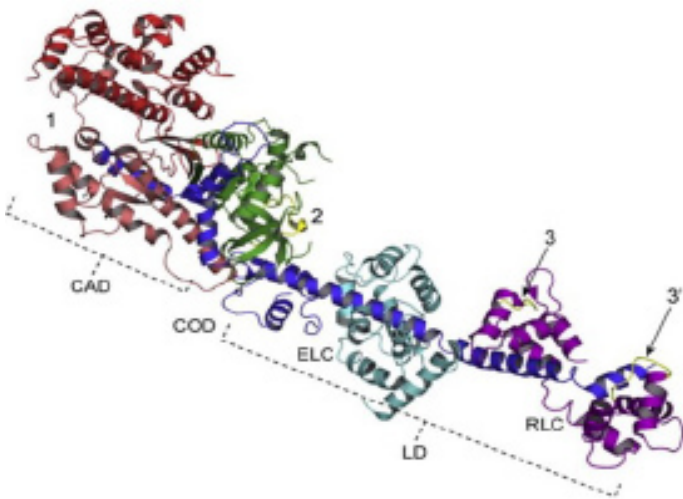


Figure 4: Diagram showing myosin head structure consisting of catalytic domain (CAD), converter domain (COD), and lever arm domain (LD). Binding sites of antibodies 1,2 and 3 are indicated by numbers 1,2 and 3 and 3', respectively [8].

Recording of myosin filament images and data analysis

Images of synthetic myosin filaments with position-marked myosin heads were recorded with an imaging plate system (IP, Fuji Photophilm, Japan) with an exposure time of 0.1sec. Under an electron microscopic magnification of 10,000X, the pixel size of the IP was 5 X 5nm. As shown in the IP records in Figure 5, the image of each gold particle was composed of a number of dark particles. The center of mass position of each gold particle was determined with an accuracy of 0.5nm, and this was taken as the position of corresponding myosin head. Care was taken to limit the total incident electron dose below 5×10^{-5} courons/cm² [9], so that the function of myosin heads was not impaired throughout each experiment. Further details of the methods have been described elsewhere [2,6].

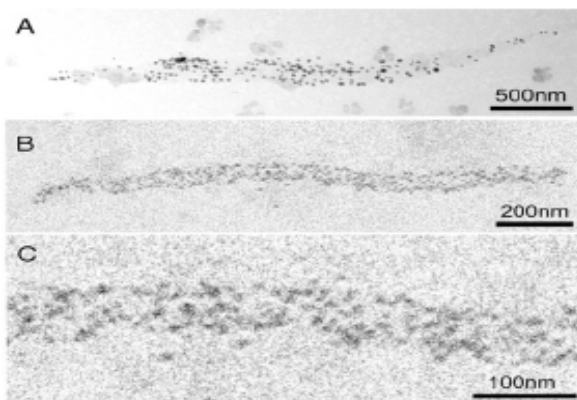


Figure 5: (A,B) Examples of IP records of spindle-shaped myosin filaments. (C) Enlarged view showing images of gold particles [2].

Results & discussion

ATP-induced myosin head movement in the absence of actin filament

Stability of myosin head position

As the first step to challenge remaining mysteries on the myosin head power and recovery strokes, we started recording myosin head movement in living myosin filaments in response to ATP application. First of all, we took a couple of IP records of the

same myosin filament with position-marked myosin heads at an interval of several min, to ascertain whether the position of individual myosin heads remains unchanged with time or not [2]. In Figure 6, open and filled circles (diameter, 20nm) are drawn around the center of mass positions of individual gold particle image, serving as the position of individual myosin heads. It can be seen that the position of each gold particle does not change appreciably with the lapse of time, so that filled circles are covered by open circles almost completely. This indicates that the time-averaged position of individual myosin heads in myosin filaments remains practically unchanged with time, providing a favorable condition to measure the ATP-induced myosin head movement [2].

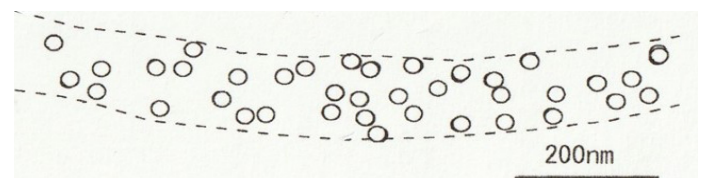


Figure 6: Stability of myosin head position in living myosin filaments. Open and filled circles (diameter, 20nm) are drawn around the center of mass positions of individual particle image in the first and the second IP records, respectively [2].

Amplitude of ATP-induced myosin head movement

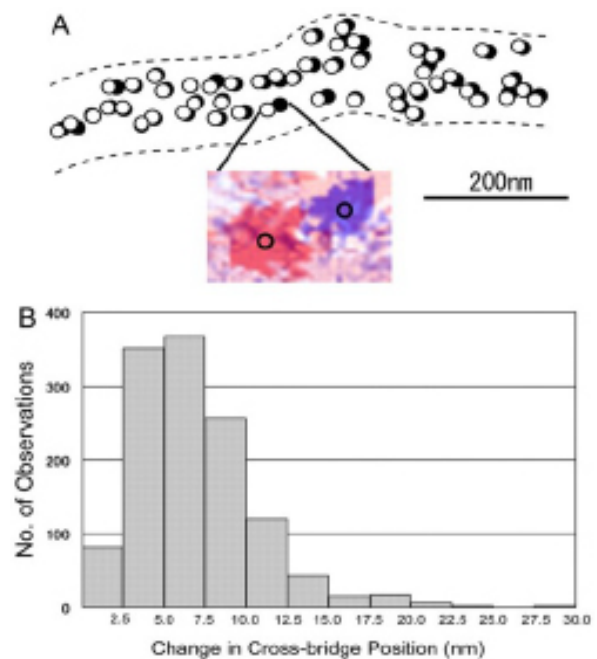


Figure 7: (A) ATP-induced myosin head movement. Open and filled circles (diameter, 20nm) are drawn around the center of mass positions of gold particles before and after ATP application, respectively. Inset is an example of gold particle profiles before (red) and after (purple) ATP application, respectively. Their center of mass positions are indicated by small circles. (B) Histogram of amplitude distribution of ATP-induced myosin head movement [2].

Being encouraged with the results indicating the stability of individual myosin heads, we applied ATP to myosin heads iontophoretically. On ATP application, all myosin heads within the electron microscopic field were found to move in one direction along the long axis of myosin filaments, as shown in Figure 7A [2]. The mean amplitude of the ATP-induced myosin head movement was ~ 6 nm (Figure 7B) [2]. In this experiment, myosin heads on myosin filament move freely in the absence of actin filaments, and would probably correspond to myosin head power stroke in muscle under free-loaded condition. These results

constitute the first direct measurement of ATP-induced myosin head movement. Unless otherwise stated, myosin heads were position-marked with gold particles using antibody 1 to the distal part of myosin head CAD.

ATP-induced myosin head movement at both sides of bare region

We could also apply ATP to myosin heads located at both sides of the bare region at the middle of myosin filament. In response to ATP, individual myosin heads were found to move away from, but not towards, the bare region, as can be seen in Figure 8. This unexpected finding indicates that, in the absence of actin filaments, myosin heads move in the direction of recovery stroke, but not in the direction of power stroke: in other words, myosin heads can decide their direction of movement without being guided by actin filaments [2]. We emphasize that this kind of unexpected finding can only be made by experiments using the EC.

In contrast with the high special resolution of IP recording (0.5mm), the time resolution of EC experiment was poor (0.1s). The reason why we succeeded in studying myosin head movement despite the poor time resolution is that the iontophoretically applied ATP concentration around myosin head was limited to a few micromole; under this condition, it takes more than 1sec for next ATP to come and bind with myosin head after completion of ATP-induced movement, so that myosin heads are fixed in position for more than 1sec after ATP application.

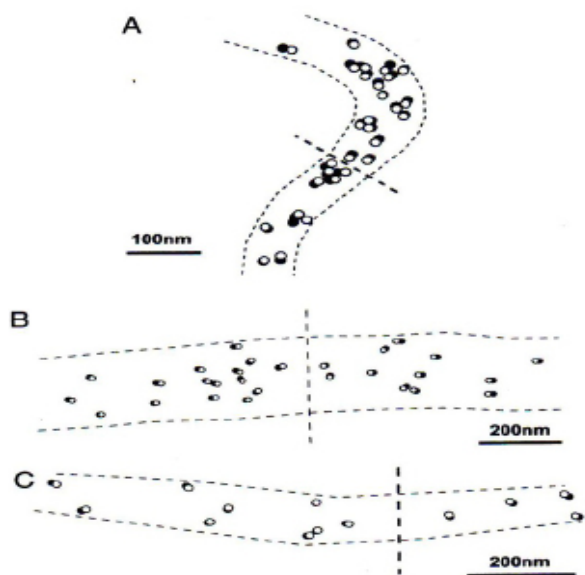


Figure 8: Examples of ATP-induced myosin head movement at both sides of the bare region (thick broken lines). Open and filled circles (diameter, 20nm) are drawn around the center of mass position of each gold particle image before and after ATP application, respectively. Note that myosin heads move away from, but not towards, the bare region [2].

Reversibility of ATP-induced myosin head movement

Figure 9 shows examples of sequential movement of nine different pixels (5 x 5nm), in which the center of mass position of gold particles are located. Pixel positions, recorded before ATP application, during ATP application, and after complete exhaustion of applied ATP are indicated by pixels colored red, blue and yellow, respectively. In A,B and I, myosin heads return to their initial position almost exactly, so that yellow pixels are not seen, being covered by blue pixels. In other records, the return of

pixels to their initial position was more or less incomplete probably due to distortion of myosin filament structure arising from ATP-induced myosin head movement. Nevertheless, the results shown in Figure 9 may be taken to indicate that, after complete exhaustion of ATP which can be facilitated by adding hexokinase and D-glucose to experimental solution, myosin heads return to their stable initial position [2].

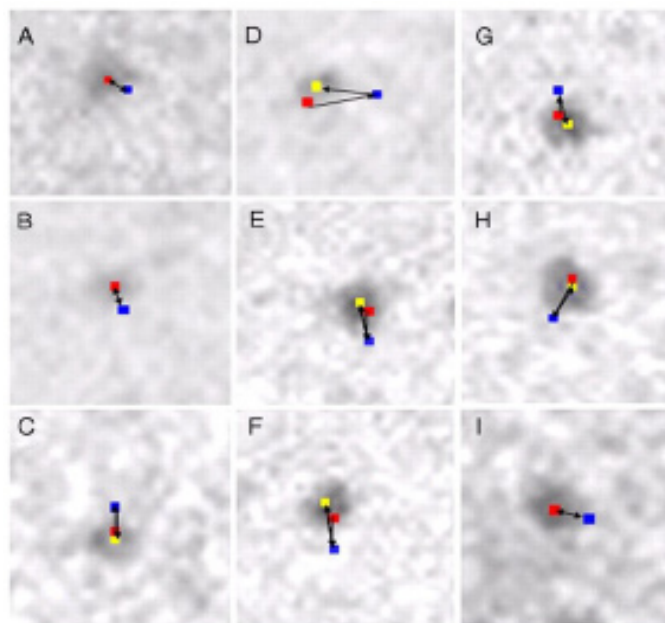
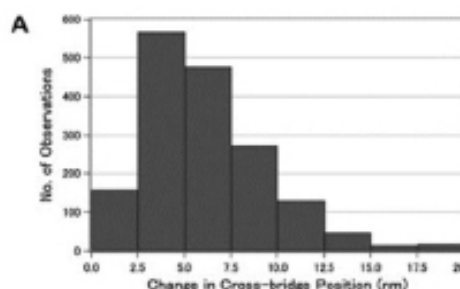


Figure 9: Examples showing sequential change in the position of different pixels, in which the center of mass positions of corresponding different gold particles are located. In each frame, pixel positions before ATP application, during ATP application, and after complete exhaustion of applied ATP are indicated by red, blue, and yellow, respectively [2].

Amplitude of ATP-induced movement at three different regions within a myosin head

To measure the amplitude of ATP-induced movement at three different regions within a myosin head, we position-marked myosin heads with antibodies 1, 2 and 3, and measured the amplitude of ATP-induced myosin head movement [8]. As shown in the histograms of amplitude distribution (Figure 10 A,B and C), the mean amplitude of ATP-induced movement at the distal region of myosin head CAD and at the proximal region of myosin head CAD was the same, being $6.14 \pm 0.09\text{nm}$ (mean \pm SD, $n=1692$) and 6.14 ± 0.22 ($n= 1112$), respectively. Meanwhile, the amplitude of movement at the myosin head lever arm domain was much smaller, being 3.55 ± 0.11 ($n=981$). As illustrated in Figure 10D, myosin head CAD remains rigid, and moves parallel to myosin filament long axis. Figure 10E is a hypothetical diagram showing possible myosin head movement in the presence of actin filaments.



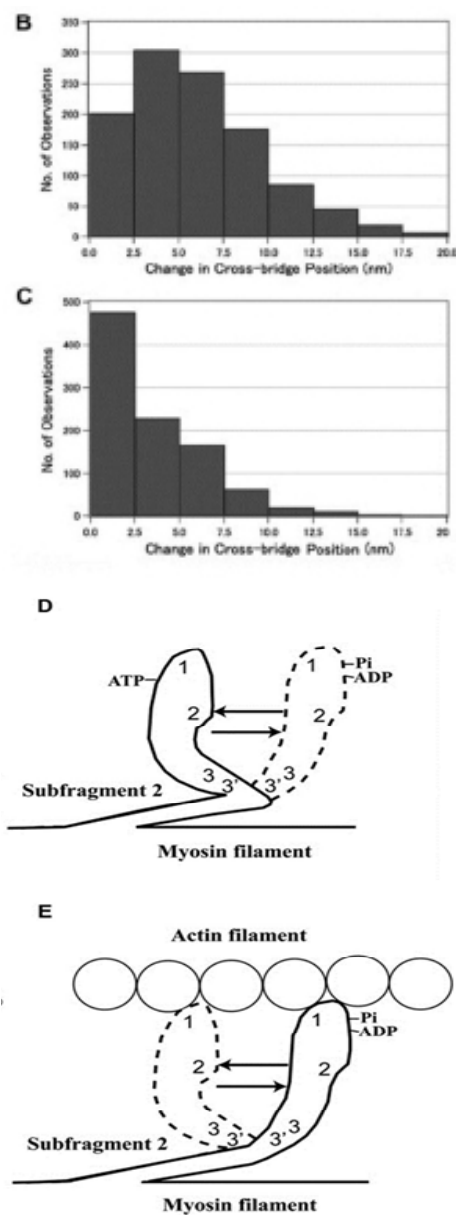


Figure 10: (A–C) Histograms of the amplitude distribution of ATP-induced myosin head movement at the distal region of myosin head CAD, at the proximal region of myosin head CAD, and at myosin head LD, respectively. (D) Diagrams illustrating myosin head conformation before (solid line) and after (broken line) ATP application. Point of attachment of antibodies 1, 2 and 3 are indicated by numbers 1, 2 and 3 and 3'. (E) The same diagram in the presence of actin filaments [8].

ATP-induced myosin head movement in the presence of actin filaments

Actin-myosin filament mixture: Its analogy with intact muscle

As the next step to investigate myosin head power and recovery strokes in the presence of actin filaments, we prepared actin-myosin filament mixture by adding actin filament sample to synthetic myosin filaments with position-marked myosin heads [10]. Figure 11 shows examples of conventional electron micrograph of actin-myosin filament mixture. It will be seen that spindle-shaped myosin filaments with position-marked myosin heads are surrounded by actin filaments. Before ATP application,

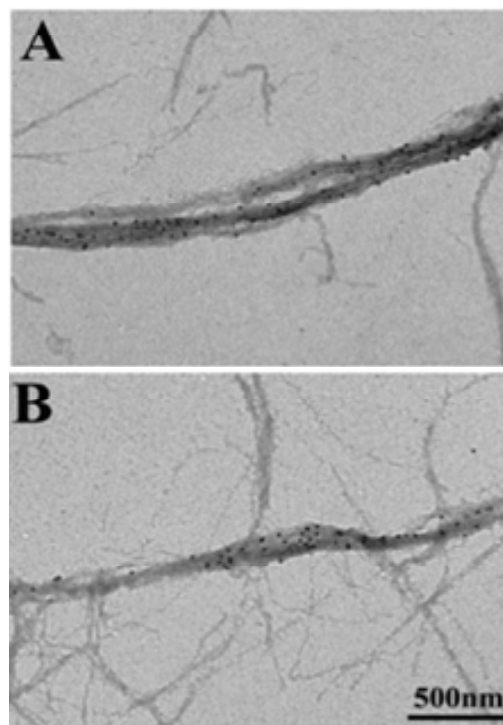


Figure 11: Conventional electron micrographs of actin-myosin filament mixture. Myosin heads were position-marked by gold particles via antibody 1 in A, and via antibody 2 in B [10].

myosin heads form rigor linkages with actin filaments, so that individual myosin heads were firmly fixed in position. Since rigor linkage have finite lifetimes up to a few min [Ishiwata et al], they break spontaneously and then again form rigor linkages with the nearest myosin-binding sites on actin filaments. Consequently, at >10min after mixing actin and myosin filaments, all myosin heads are expected to form rigor linkages with actin filaments without producing rigor forces.

On ATP application, myosin heads bind with ATP to detach from actin filaments, as $A-M + ATP \rightarrow A + M-ATP$, and then hydrolyze ATP to form $M-ADP-Pi$ to start cyclic interaction with actin filaments by repeating power and recovery strokes. Therefore, the response of myosin heads in the actin-myosin filament mixture to applied ATP may be analogous to what takes place in living muscle.

Two different modes of myosin head power stroke depending on experimental conditions

As already mentioned, the ATP concentration around myosin heads does not exceed a few μM , and only a small fraction of myosin heads in

Myosin filaments are activated to react with actin filaments, while majority of myosin heads form rigor A-M linkages. For this reason, activated myosin can not produce gross sliding between actin and myosin filaments, but only move for a limited distance by stretching adjacent elastic structures. As a matter of fact, in the standard ionic strength of experimental solution (125mM), the mean amplitude of ATP-induced myosin head power stroke was $3.3 \pm 0.2nm$ (mean \pm SD, $n=732$) at the distal region, and $2.5 \pm 0.1nm$ ($n=613$) at the proximal region of myosin head CAD [10]. Myosin heads returned to their initial position after complete exhaustion of applied ATP.

In the low ionic strength (20mM), which enhances Ca^{2+} -activated force generation in skinned skeletal muscle fibers twofold [11], the mean amplitude of ATP-induced power stroke

increased to $4.4 \pm 0.1 \text{ nm}$ ($n=361$) and $4.3 \pm 0.2 \text{ nm}$ ($n=305$) at the distal and the proximal regions of myosin head CAD, respectively.

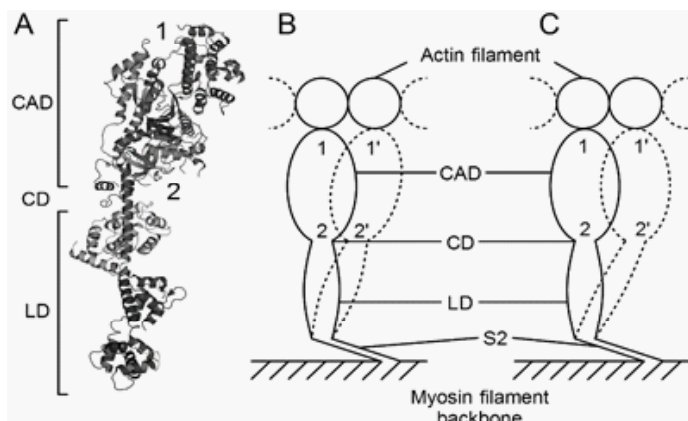


Figure 12: Change in the mode of myosin head power stroke depending on ionic strength of experimental solution. (A) Diagram of myosin head structure consisting of catalytic (CAD) and lever arm (LD), connected with small converter (COD) domain. Numbers 1, 2 indicate approximate points of attachment of antibodies 1 and 2, respectively. (B) The mode of myosin head power stroke at standard ionic strength. Amplitude of stroke is larger at distal region than at proximal region of CAD. (C) The mode of myosin head power stroke at low ionic strength. Amplitude of stroke is the same in both distal and proximal regions of CAD [9].

In all the EC experiments performed, the image of gold particles attached to myosin heads were clearly recorded on the IP, so that the particle images, recorded before and after ATP application were indistinguishable. This indicates that, as with myosin heads before ATP application, the time-averaged myosin head mean position is stable and does not change with time.

Conclusion

Although myosin head power and recovery strokes, producing muscle contraction, are explained in every textbook in physiology as if they are established facts, they remained a mystery without experimental evidence over many years. Using the EC, we made it possible to observe position-marked individual myosin head in living muscle myosin filaments, under an electron microscope, thus constituting the first success in making a long-lasting dream of biological investigators come true.

The novel properties of ATP-induced myosin heads, which have been revealed by our EC experiments are summarized as follows: (1) In relaxed state, the time-averaged myosin head position remains unchanged despite thermal agitation; (2) In the absence of actin filaments, myosin head move in the direction of recovery stroke, i.e. away from the bare region of myosin filament, in response to ATP application; (3) The mean value of ATP-induced myosin head movement is $\sim 6 \text{ nm}$; (4) after complete exhaustion of applied ATP, myosin heads return to their initial position; (5) In the actin-myosin mixture, in which only a small fraction of myosin heads can be activated by ATP application, myosin heads exhibit two different modes of power stroke depending on the ionic strength; (6) In both the presence and the absence of actin filaments, the time-averaged position of myosin heads, which had moved in response to ATP, is stable; and (7) After completion of power stroke, myosin heads return to their initial position on complete exhaustion of applied ATP.

It is our pleasure that techniques of “liquid cell electron microscopy” are developing in the field of materials science [15],

so that investigators can now study physical and chemical reactions in water electron microscopically. Though our experimental work using the EC was terminated 10 years ago, due to difficulties arising mainly from my old age (86), I heartily hope that the EC methods developed by us will be widely used by physiologists and biologists in future to open new horizons in every field of biological science.

Acknowledgements

We wish to dedicate this article to the late Professor Akira Fukami, who developed the EC system, without which our work was not achieved. Our thanks are also due to President Kazuo Ito of Japan Electron Optics Laboratory, Inc. (JEOL) for his generous support.

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