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Bi-directional movement of actin filaments along long bipolar tracks of oriented rabbit skeletal muscle myosin molecules

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Abstract In actomyosin in vitro motility assays, orientation of myosin molecules affects their interaction with actin. We obtained long tracks of myosin molecules with uniform orientation. Bipolar filaments about 50 μm long were made from myosin rod prepared from molluscan smooth muscles, to which rabbit skeletal-muscle myosin bound, creating long synthetic thick-filaments. Movement of F-actin toward their center was much faster (4.7 ± 0.6 μm s^{-1}) than in the opposite direction (1.9 ± 0.2 μm s^{-1}), indicating that myosin molecules were arranged in the same orientation along each half of the bipolar filament. These complex thick-filaments permit measurement of actin movement over 20 μm of oriented skeletal myosin tracks facilitating mechanistic studies of actomyosin motility.

Key words: Myosin; Actin; In vitro motility assay; Myosin rod; Molluscan smooth muscle

1. Introduction

The in vitro motility assay system is very useful for studying the mechanism of energy conversion by motor proteins, such as myosin, dynein and kinesin. In this system, motor protein is attached to a glass surface and fluorescent actin filaments or microtubules moving on the surface in the presence of ATP can be observed by a light microscope [1–4]. In most cases, motor protein molecules are attached to the glass surface in random orientations, and moving actin filaments or microtubules interact with motor molecules in many different orientations. Movement assays of actin filaments along large native thick filaments of molluscan smooth muscles have shown that the orientation of myosin molecules is very important for the movement [5,6]. Myosin molecules in each half of bipolar thick filaments are considered to be arranged in the same orientation [7]. Although the direction of movement is determined by the polarity of actin filaments [2,8], myosin molecules in the wrong orientation with respect to the normal interaction between thick and thin filaments in vivo, can still move actin filaments in vitro; however, actin velocity is much slower and the affinity between actin and myosin is much less compared with correctly-oriented myosin [5,6,9]. Also, synthetic filaments of purified rabbit skeletal muscle myosin show similar results [10–12]. However, since these were about 5 μm long, the length of myosin track with uniform orientation was only about 2–3 μm. Therefore, the steady-state movement under unloaded conditions by correctly-oriented myosin molecules only lasted for less than one second. In order to study the movement by correctly-oriented myosin molecules, it is important to obtain much longer tracks of oriented myosin molecules.

In this report, we describe a method for making tracks of oriented rabbit-skeletal muscle myosin molecules whose lengths are more than 20 μm. We used large synthetic filaments of myosin rods made from molluscan smooth muscles as backbones for rabbit myosin tracks. The velocities of actin filaments toward, and away from, the center of these complex filaments were comparable to those observed for the synthetic myosin filaments reported previously [10].

2. Materials and methods

2.1. Proteins

Myosin was extracted and purified from rabbit leg and back fast-twitch muscles essentially as described by Szent-Gyorgyi [13]. Actin was purified from rabbit-skeletal muscle acetone-powder as described by Spudich and Watt [14].

A high salt extract was prepared from the anterior byssus retractor muscle (ABRM), the posterior byssus retractor muscle (PBRM) and the posterior adductor muscle (PAM) of *Mytilus edulis* essentially described by Castellani and Cohen [15]. As described by Castellani et al. [16], this extract contained considerable amount of myosin rod protein (see Section 3 and Fig. 1, lane 1). After the extraction, a 30–42% $(NH_4)_2SO_4$ fraction, instead of the 37.5–60% fraction [15], was collected to obtain higher yield of myosin rod. Three volumes of ethanol were added to the extract, and the precipitated material resuspended in 0.4 M KCl, 2 mM EDTA, 2 mM dithiothreitol (DTT), 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES)-KOH (pH 7.0). After incubation on ice for 1 h with extensive stirring, followed by centrifugation at 350000 × *g* (Beckman, TLA-100.2 rotor) for 2 h, the supernatant was collected. Myosin rods formed filaments on lowering KCl concentration to 0.1 M, and were collected by centrifugation at the same speed for 10 min. By this method, about 1 mg of the rod protein could reproducibly be obtained from 0.5 g of muscles.

Protein concentration was determined spectrophotometrically using a micro BCA protein assay kit (Pierce Chemical Co. Ltd.), using bovine serum albumin (BSA) as a standard. Analysis by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out under the discontinuous buffer system of Laemmli [17]. The gel was stained with Coomassie brilliant blue R-250.

2.2. Electron microscopy

Low-angle rotary-shadowed samples of the purified protein at high ionic concentration, 0.4 M KCl, 2 mM EDTA, 2 mM DTT, 10 mM PIPES-KOH (pH 7.0) were obtained essentially according to Tyler and Branton [18]. The samples were observed on a JEOL JEM 2000-EX electron microscope at an accelerating voltage of 80 kV.

2.3. Partial amino acid sequencing

Four volumes of cold water was added to the crude muscle extract without $(NH_4)_2SO_4$ fractionation (see Fig. 1, lane 1), and centrifuged

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Abbreviations: PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide electrophoresis; BSA, bovine serum albumin; DTT, dithiothreitol; ABRM, anterior byssus retractor muscle; PBRM, posterior byssus retractor muscle; PAM, posterior adductor muscle

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at 20000×*g* (Hitachi, RPR 20-2 rotor) for 30 min. The precipitate, containing myosin, the 120-kDa polypeptide, and the 112-kDa polypeptide was subjected to SDS-PAGE. The gels were briefly stained with Coomassie brilliant blue R-250 and the bands of the three polypeptides were cut out. Each denatured polypeptide within the cut gels was electrophoretically collected and treated with 20 mM *o*-iodosobenzoic acid in the presence of 0.1 M HCl, 20 mM *p*-cresol and 75% acetic acid overnight at room temperature. This treatment causes cleavage of polypeptide chains mainly at the carboxy group of tryptophan residues [19]. The resultant peptide fragments were separated by the SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane. Some bands were cut out and analyzed on an amino acid sequencer (Applied Biosystems, 473A).

2.4. Preparation of myosin tracks and motility assay

Large filaments were obtained from the myosin rod (0.1 mg protein ml^{-1}) by lowering ionic concentration slowly, as described previously [10]. The resulting filament suspension in 0.1 M KCl, 2 mM EDTA, 2 mM DTT, 10 mM PIPES-KOH (pH 7.0) was rapidly mixed with 1/9 volume of 0.1 mg ml^{-1} rabbit skeletal muscle myosin dissolved in 0.4 M KCl, 2 mM EDTA, 2 mM DTT, 10 mM PIPES-KOH (pH 7.0), and was left on ice for 1 h so that myosin could bind to the filaments. The large filaments were collected by centrifugation at 1500×*g* (Kubota, RA-53G rotor) for 20 min and then resuspended in the 0.1 M KCl solution. Unbound myosin, which might form small filaments, remained in the supernatant.

In vitro motility assay experiment was performed essentially according to Kron and Spudich [2]. The flow cell was constructed with silicone-coated coverslips [20]. The inside of the cell was first treated with 0.05 mg ml^{-1} BSA for 2 min. The complex filament suspension was then perfused to bind the filaments to the glass surface. After unbound filaments were washed out, actin filaments (10 nM in monomer) labeled with rhodamine-phalloidin (Molecular Probes) in 5 mM $MgCl_2$, 2 mM ethyleneglycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA), 1 mM Na_2ATP, 10 mM DTT, 20 mM HEPES-KOH (pH 7.8), 5 mg ml^{-1} glucose, 50 μg ml^{-1} glucose oxidase, 10 μg ml^{-1} catalase were introduced. Actin movement was observed using a Nikon TMD inverted light microscope with a high sensitivity SIT television camera (Hamamatsu Photonics, C2400-08) at 28°C.

3. Results and discussion

3.1. Preparation of Mytilus myosin rod

Castellani et al. [16] reported that crude extract of the *Mytilus* ABRM contained considerable amounts of myosin rod whose apparent molecular weight determined by the SDS-PAGE was 110k. We purified this in order to use filaments of rods as backbones for arranging rabbit myosin molecules. In addition to the ABRM, we used the PBRM and the PAM for its preparation, since their extract also contained rods (data not shown). Fig. 1 shows the changes in the protein composition revealed with the SDS-PAGE during the purification procedure. The high salt muscle extract contains the 120-kDa and the 112-kDa polypeptides which were considered to correspond the protein that had been reported, as well as the myosin heavy chain, paramyosin, actin, tropomyosin, the myosin light chains, and other minor proteins (lane 1). After $(NH_4)_2SO_4$ fractionation, most of the actin and paramyosin were removed (lane 2), but the two peptides remained. The myosin heavy chain was removed following ethanol denaturation and renaturation, although slight amounts of the myosin light chains and tropomyosin seemed to remain (lane 3). When the KCl concentration was lowered to 0.1 M, the myosin rod formed filaments which could be collected by centrifugation and the other remaining proteins were removed (lane 4).

We observed the final product containing two peptides of 120 kDa and 112 kDa under high ionic concentrations (0.4M KCl) by electron microscopy of low-angle rotary-shadowed samples. Rod-shaped structures with a length of about 150 nm were observed (Fig. 2), indicating the fraction contained the myosin rod.

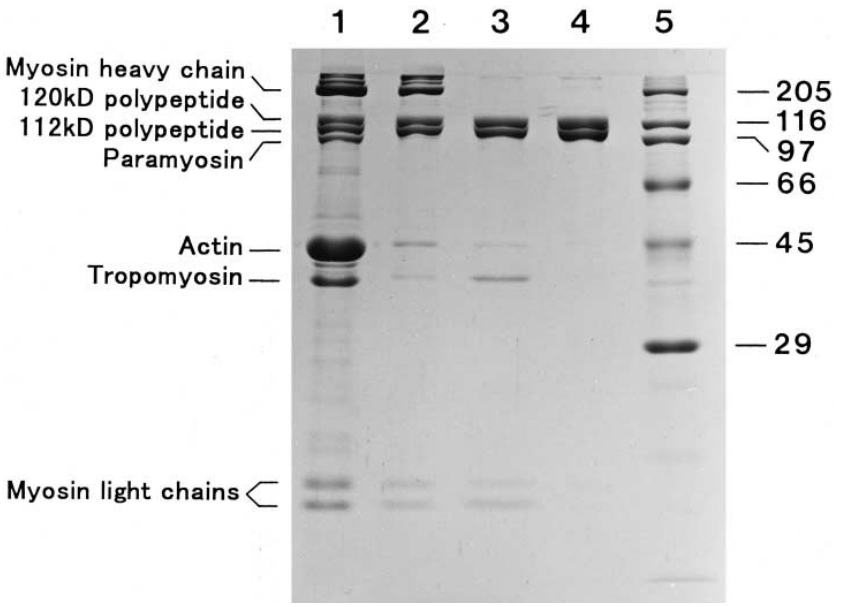

Fig. 1. Protein compositions shown by the 12.5% SDS-PAGE of intermediate and final stages of purification. Lane 1 shows the high salt extract of the muscles, which contains myosin, the 120-kDa and the 112-kDa polypeptides which were considered to be components of myosin rod, paramyosin, actin and tropomyosin. Ammonium sulfate fractionation removes paramyosin and most actin and tropomyosin (lane 2). After ethanol denaturation, the myosin heavy chain was completely removed by centrifugation (lane 3). On lowering the ionic concentration, the rod formed filaments and could be collected (lane 4). Lane 5 shows the molecular weight protein marker mixture (Sigma, SDS-6H) with molecular masses (kDa) on the right.

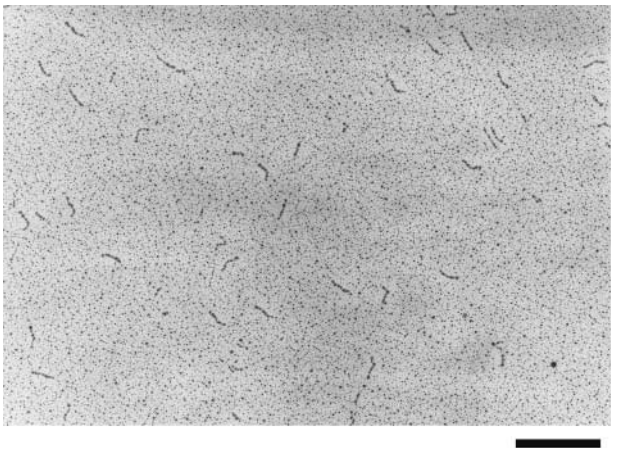

Fig. 2. Electron microscopic observation of the final product at high ionic strength. The specimen was prepared by the rotary shadowing technique [18]. The rod-shaped structures about 150 nm in length suggest that the protein in the final product was the myosin rod. The bar indicates 0.5 μm.

Castellani et al. [16] reported the apparent molecular weight of the polypeptide of the *Mytilus* myosin rod was about 110k. Since the final product of our preparation consists of two polypeptides whose apparent molecular weights were 120k and 112k, either of these may be a contaminating polypeptide, or both may be components of the myosin rod. We compared partial amino acid sequences of three polypeptides, i.e. the myosin heavy chain (about 220 kDa), the 120 kDa polypeptide and the 112-kDa polypeptide. After the three polypeptides were isolated and cleaved with *o*-iodosobenzoic acid, the resulting fragments were separated by the SDS-PAGE. All three polypeptides produced, among their cleavage fragments, two fragments running at apparent molecular masses of 68 kDa and 58 kDa on SDS-PAGE gels (data not shown). We analyzed the N-terminal amino acid sequences of these six fragments. The three 68-kDa fragments showed the same sequence, RVKVESEGANKAEELEEARR, and the three 58-kDa fragments showed the same sequence, QAKVTDLQSELENAQKEA. These sequences from *M. edulis* are comparable to the known amino acid sequences of molluscan striated-muscle myosin heavy-chain from *Argopecten irradians* (previously *Aequipecten irradians*), RSKFESEGANRTEELEDQKR (1369–1388) and QAKVNSLQSELENSQKES (1454–1471) [21]. This indicates that both the 120-kDa and the 112-kDa polypeptides were the components of the myosin rod.

During the course of the rod protein preparation, we found that the ratio of the quantities of rod protein and the myosin heavy chain in the muscle homogenate did not change from preparation to preparation, and remained constant even after the muscle homogenate was left overnight without protease

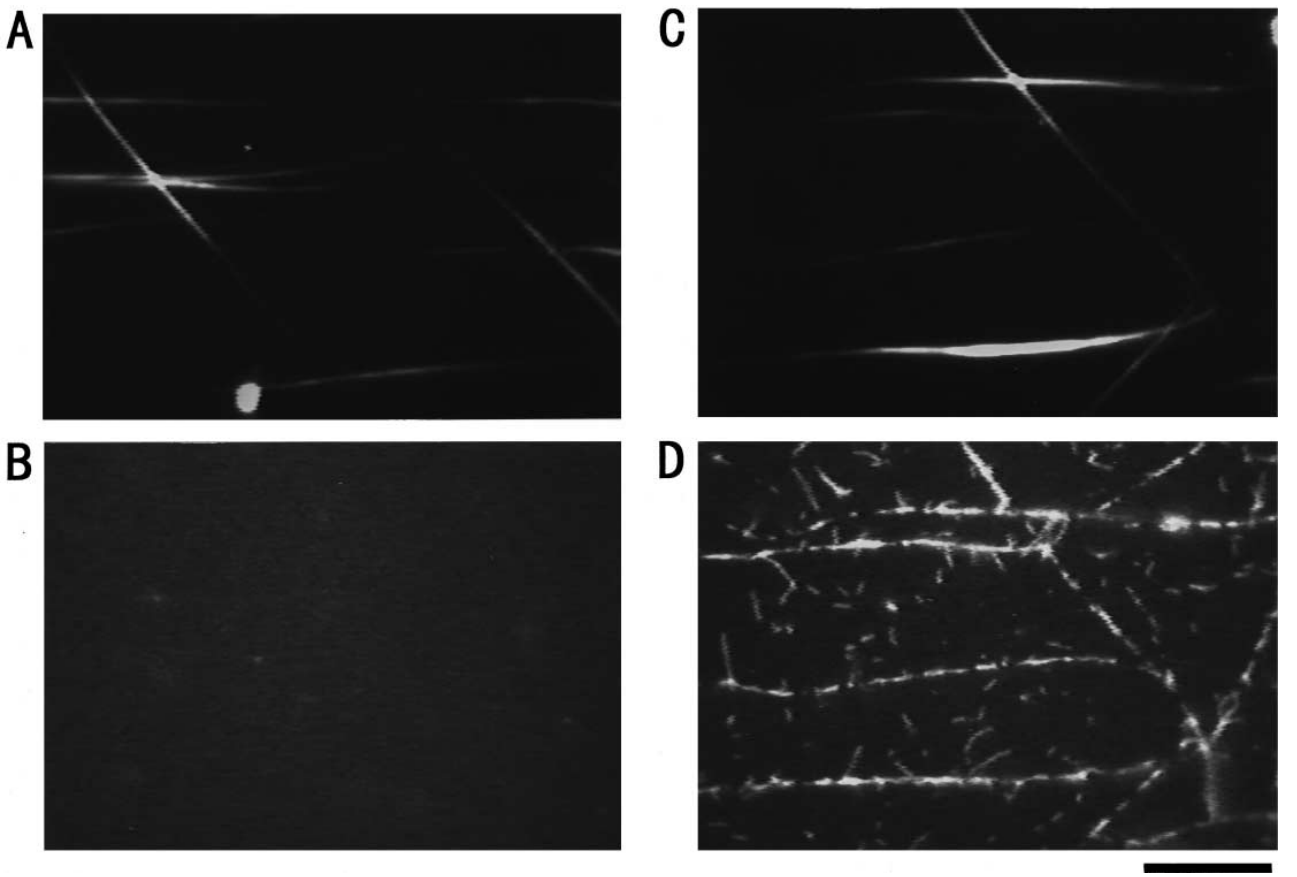

Fig. 3. Binding of actin filaments to the rod filaments before and after binding rabbit myosin. Either the rod filaments without rabbit myosin (A, B) or those which had been treated with rabbit myosin (C, D) were bound to the glass surface and then rhodamine-labeled actin filaments were added in the absence of ATP. The glass surface was observed by dark-field illumination showing the rod filaments (A, C) and by fluorescence illumination showing rhodamine-labeled actin filaments (B, D) in the same field of view. All photographs are in the same magnification and the bar indicates 10 μm.

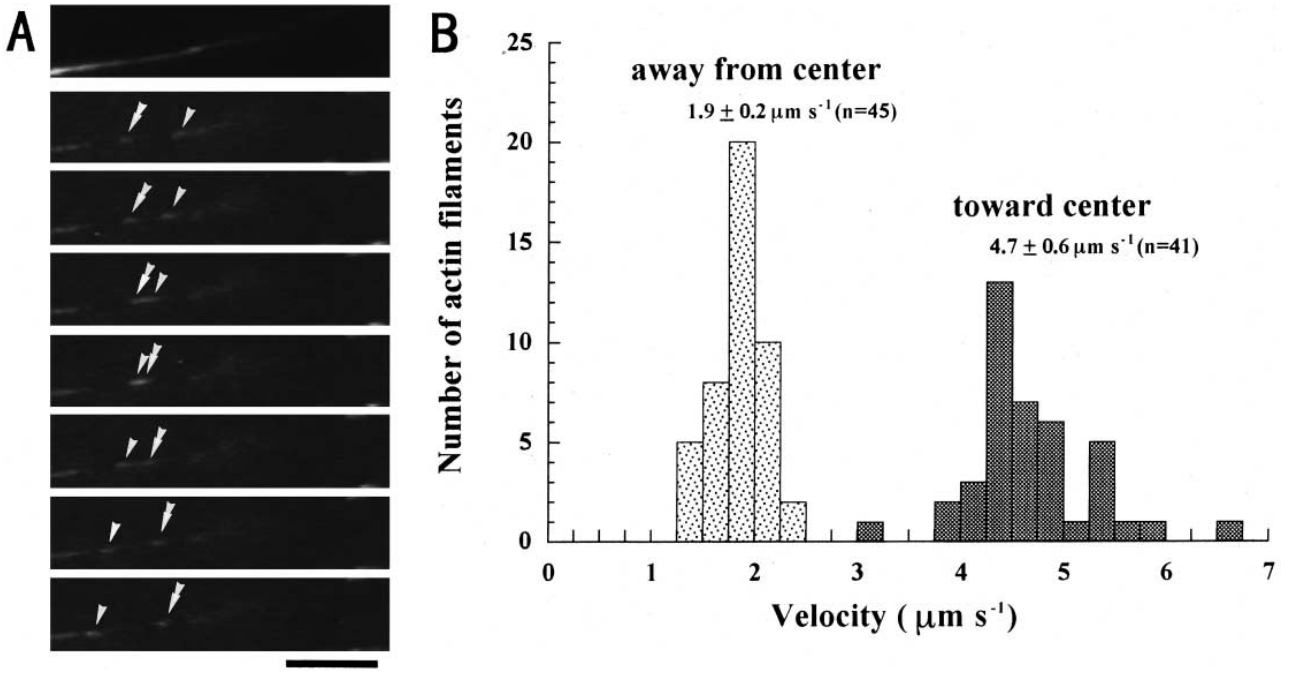

Fig. 4. Movement of actin filaments along myosin tracks formed on the rod filaments. (A) An example of movement of two actin filaments along the right half of one rod filament to which rabbit myosin had been attached. The uppermost photograph is the dark-field image showing the rod filament. The position of its center is near the left side of the image. The other 7 photographs show the fluorescence images taken with 0.2 s intervals. The actin filament indicated by arrowheads moved toward the center of the rod filament and the other indicated by double-arrowheads moved in the opposite direction. Note that the former moved much faster than the latter. All photographs are in the same field of view with the same magnification, and the bar indicates 5 μm. (B) The distribution of the velocities of the actin filaments moving toward, and away from, the center of the rod filaments.

inhibitors at room temperature (data not shown). In addition, the N termini of both polypeptides of the rod protein might be modified in vivo since we failed to determine their N-terminal amino acid residues by the Edman degradation method. These facts suggest that the rod protein was been produced in living muscle cells, rather than that they formed after the muscles were dissected from the animals. In *Drosophila* muscles, myosin rod protein without a motor domain is produced from the myosin heavy chain gene by an alternative promoter [22]. Whether the present rod protein is produced in *M. edulis* muscles in a similar manner is being investigated.

3.2. Movements of actin filaments along myosin tracks

On lowering ionic concentration slowly, myosin forms relatively large filaments. In case of the *Mytilus* myosin rod, filaments about 50 μm in length were formed, and could easily be observed by a dark-field light microscope (see Fig. 3A). To see whether they had any affinity for actin filaments, we introduced them into the flow cell used for the motility assay experiment, and added rhodamine-labeled actin filaments in the absence of ATP. The rod filaments attached to the glass surface could be observed under the dark-field illumination (Fig. 3A), but fluorescence microscopic observation in the same field of view revealed that actin filaments did not bind to the rod filaments (Fig. 3B). We carried out the same experiments but using the rod filaments to which rabbit skeletal muscle myosin had been bound. Comparison between the dark-field image and the fluorescence image in the same field of view shows that actin filaments bound to the rod filaments (Fig. 3C and D). We consider that the myosin molecules attached to the rod filaments made rigor complexes with the rhodamine-labeled actin filaments. There were also actin filaments attached to the glass surface where rod filaments were absent. This may be because myosin that had not been attached to the rod filaments remained and bound to these areas of the surface. From these results, we concluded that the rod filaments have no affinity for actin filaments, but they could bind rabbit skeletal muscle myosin molecules to form complex filaments which were then able to bind actin.

We observed the movement of rhodamine-labeled actin filaments along the myosin tracks made on the rod filaments in the presence of 1 mM ATP. Fig. 4A shows two actin filaments moved on the same track, but in the different directions, with the dark-field image of the same field of view. One moved toward the center of the rod filament (arrowheads) was much faster than the other (double-arrowheads). We examined the velocities of the two types of movement, i.e. in the direction toward the center of the rod filaments and in the opposite direction at 28°C (Fig. 4B). The former was 4.7 ± 0.6 μm s^{-1}(mean ± SD, $n = 41$), and the latter was 1.9 ± 0.2 μm s^{-1} ($n = 45$). These values are comparable to those observed on filaments reconstituted only from purified rabbit myosin [10]. This indicates that myosin molecules were arranged in the same orientation along the half of each rod filament as along that of each filament reconstituted from purified rabbit myosin alone. The bipolar arrangement of the rabbit skeletal muscle myosin strongly suggests that the backbone filaments made of the *Mytilus* myosin rod were also bipolar, and that the rabbit myosin molecules could bind by side-by-side interaction only in a parallel oriented manner, but not in an antiparallel orientation. It should be noted that the change in actin velocity, observed with rabbit skeletal muscle myosin between correctly and wrongly oriented myosin [10,12], which is also presented here (Fig. 4), is not as great as that for native

molluscan thick filaments [5,6]. This may be due to differences in the myosins. Also the reconstituted system is not as clean as native thick filaments, thus this smaller velocity change could be due to a minor number of incorrectly-oriented myosin molecules being present in the reconstituted system. Using a similar reconstituted system, A. Ishijima and T. Yanagida (personal communication) tried to arrange rabbit myosin molecules on clam paramyosin filaments. But they did not find any difference between the velocities of actin filaments moving in the two directions. Paramyosin filaments may not be useful for arranging myosin molecules in the same orientations.

Much knowledge about the mechanism of actomyosin energy conversion has been obtained using in vitro motility assay experiments. Most of these experiments were not performed with controlled orientation of the motor protein molecules. However, their orientation affects motility and should be controlled. Molluscan native thick filaments [5,6,9] and synthetic myosin filaments [10–12] have been used for experiments with oriented myosin molecules, but the latter are often so short that the movement of actin filaments on them cannot easily be observed. The tracks presented here are much longer than those of the synthetic filaments of purified rabbit myosin alone, and would be useful for in vitro motility experiments, especially in which movement over long distances needs to be observed. Imafuku et al. [23] reported that in the in vitro motility assay, the fluctuation in the translational movement of microtubules or actin filaments was independent of their length. A possible explanation is to consider the heterogeneity in the distribution of motor protein on the glass surface in the motility assay system. They said that molluscan native thick filaments would be useful for checking this possibility. The present system enables such experiments using rabbit skeletal muscle myosin in addition to using molluscan native thick filaments. Furthermore, myosin II from other sources which can bind to the present long rod filaments will be able to be used for experiments with controlled orientation of motor protein molecules.

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