J. Biochem. **138**, 255–262 (2005)
DOI: 10.1093/jb/mvi121

Biochemical Properties of Ordinary and Dark Muscle Myosin from Carp Skeletal Muscle

Tsuyoshi Okagaki*, Masaki Takami, Kiyo Hosokawa, Miyuki Yano, Sugie Higashi-Fujime and Atsushi Ooi

Department of Bioresources, Mie University, Kamihama 1515, Tsu, Mie 514-8507

Received January 26, 2005; accepted May 31, 2005

Two types of myosin isolated from ordinary (fast) and dark (slow) muscles of carp were examined by ATPase and *in vitro* motility assays. V_{max} of the ATPase activity and sliding velocity of ordinary myosin showed 1.6 and 1.5 times higher activities than those of dark myosin, whereas those of mammalian fast myosin were much higher, 3 to 10 times, than those of slow myosin. Although ordinary myosin had almost identical activities to those of mammalian fast myosin, activities of dark myosin was twice of those of mammalian slow myosin. This high motile activity of dark myosin can account for the physiological role of dark muscle in cruising of fish. By comparing K_m of the actin-activated ATPase activity, ordinary myosin was appeared to have higher affinity to F-actin than dark myosin, and this was confirmed by the binding assay of HMM or S-1 of carp myosin to F-actin. Investigation of myosin assembly by electron microscopy and the centrifugation assay revealed that ordinary myosin assembled much poorly than dark myosin or mammalian fast myosin. This phenomenon may reflect characteristic cellular function of fish skeletal muscle.

Key words: fast muscle, fish myosin, motility assay, myosin filament, slow muscle.

Abbreviations: F-actin, filamentous actin; HMM, heavy meromyosin; S-1, subfragment 1; PMSF, phenylmethanesulfonyl fluoride.

Vertebrate skeletal muscle is classified into two types, fast and slow muscle, based on its shortening velocity (*1*, *2*). Fast muscle works mainly in an aerobic metabolism, and generates a short but strong contraction force. Slow muscle generates a slow twitch and continuous contraction for a long period. The contraction force of muscle is basically driven by the ATP-dependent sliding movement between thick and thin filaments, composed of myosin and actin, respectively. The magnitude of the contraction force closely depends on the mechanochemical activity of myosin. The ATPase activity of myosin from fast muscle is higher than that from slow muscle, reflecting its faster shortening velocity. The heavy chains of fast and slow myosin have different amino acid sequences (*3*, *4*).

Fish also have two types of muscle, ordinary and dark, which correspond to the fast and slow muscles in mammals or birds (*5–7*), respectively. Ordinary muscle occupies a large proportion of the body, whereas dark muscle is localized just under the lateral line. This localization is quite different from that of mammalian fast and slow muscles. Carp have three different myosin heavy chain genes in ordinary muscle. Gene expression of these three genes switches depending on changes in body temperature due to environmental conditions, because these myosin isoforms have different thermal stabilities (*8–11*).

In addition to the difference in amino acid sequence between ordinary and dark myosin (*12*), ordinary myosin includes three types of light chains, while dark muscle has a different set of two types of light chains (*13–16*). The biochemical properties of ordinary and dark muscle from several species of fish, including mackerel and yellowtail, were previously examined, and it was shown that the myosin ATPase activities of ordinary myosin are higher than those of dark myosin, as in the case of mammalian fast and slow myosin (*5*, *6*).

These two types of myosin in fish, however, have not been well characterized, but knowledge about their precise properties is needed in order to understand the physiological roles of ordinary and dark muscle. Therefore, we undertook an extensive examination of the mechanochemical properties of these two types of myosin by *in vitro* motility (*17*, *18*) and ATPase assays, and also by analyzing self-assembly of myosin. Dark myosin has much higher mechanochemical activities than mammalian slow myosin, and ordinary myosin has a lower ability to form filaments than mammalian fast myosin. These properties may be favorable for fish cruising in water, and also for the quick turnover in myosin molecules upon switching the expression of myosin heavy chain genes.

MATERIALS AND METHODS

Purification of Proteins—Live specimens of carp, *Cyprinus carpio*, cultivated at 14–17°C were purchased from Marusan Fisheries Co., Ltd. (Tsu-city, Mie, Japan), and brought to our laboratory. After the fish were killed, the dorsal muscle was collected and chilled on ice for about 10 min, and then ordinary and dark muscle were carefully separated. Myosin was prepared from these two types of muscle according to the method reported previ-

*To whom correspondence should be addressed. Tel: +81-59-231-9723, Fax: +81-59-231-9723, E-mail: okagaki@bio.mie-u.ac.jp

ously (*19*) with slight modifications. In the last step of the preparation, dark myosin was collected by centrifugation at 10,000 × *g* for 20 min at a low concentration of KCl. Due to the poor ability to form myosin filaments (see "RESULTS"), ordinary myosin did not sediment completely under the same conditions. Therefore, ordinary myosin was collected by centrifugation at 100,000 × *g* for 20 min. The precipitated myosin was suspended in a solution containing 0.6 M KCl, 40 mM Tris-HCl (pH 7.5), 2 mM DTT and 4 mM EDTA, and clarified by centrifugation at 10,000 × *g* for 20 min. The supernatant was thoroughly mixed with an equal volume of cold glycerol, and stored at –30°C, or aliquots were frozen in liquid nitrogen and kept at –85°C for long term storage. Actin was prepared from acetone dried carp ordinary muscle as described (*20*), and purified actin was kept on ice in F-buffer (0.1 M KCl, 0.1 mM $MgCl_2$, and 0.1 mM ATP).

Motility and ATPase Assays—*In vitro* motility assays were performed at 25°C according to Okagaki *et al.* (*21*, *22*) in motility assay buffer containing 1 mM ATP, 2 mM $MgCl_2$, 25 mM DTT, 30 mM Imidazole (pH 7.6), 0.216 mg/ml glucose oxidase, 0.036 mg/ml catalase, 4.5 mg/ml glucose, and various concentrations of KCl. Myosin in the filamentous form (0.22–0.33 μM) in a solution containing 0.05 M NaCl and 20 mM Tris-HCl (pH 7.5), was immobilized on a glass coverslip coated with nitrocellulose. Then, the coverslip was combined with a glass slide to form a flow cell. Rhodamine-phalloidin labeled F-actin in motility assay buffer was introduced into the flow cell, and the sliding movement of the actin filament was observed under an epifluorescence microscope (Olympus, BH2) equipped with a SIT camera (Hamamatsu Photonics, C2400). Actin activated Mg^{2+}-ATPase activity was assayed at 25°C by the method of Hayashi (*23*) in 30 to 80 mM KCl, 20 mM HEPES (pH 7.6), 1 mM ATP, 2 mM $MgCl_2$ and 0.11 μM myosin by varying the concentration of actin from 0.23 to 4.8 μM. The V_{max} and K_m of the ATPase activity were calculated from double reciprocal plots by the mean least squares method.

Preparation of Myosin Fragments, HMM and S-1, and Binding Assay of the Fragments to F-Actin—Myosin was cleaved with α-chymotrypsin according to the procedure of Kato and Konno (*24*), and HMM and S-1 were separated by DEAE ion exchange column chromatography. For binding assays, HMM and F-actin were mixed at concentrations of 1.1 μM and 12 μM, respectively, in 20 mM Tris-HCl (pH 7.5), 1 mM ATP, 2 mM $MgCl_2$ and 0.1 M KCl and incubated on ice for 30 min. Then the mixture was centrifuged at 100,000 × *g* for 40 min at 4°C. After the supernatant and precipitate were subjected to SDS-PAGE, the gel was stained with Coomassie Brilliant Blue, and the bands of polypeptides in each lane were densitometrically scanned and analyzed by NIH image (ver 6.0). Binding of S-1 to F-actin was also performed under the same experimental conditions as described above. We calculated the amounts of bound proteins from the molecular masses as follows: actin 42 kDa, HMM 330 kDa, and S-1 120 kDa.

Microscopic Observations of Myosin and Rod Filaments—For observation of filament structures, myosin (0.22 μM) or rods (0.11 μM) were assembled in 0.15 M KCl, 20 mM HEPES (pH 7.4), 1 mM DTT and 1 mM EDTA for 30 min. The specimen was stained with 1% uranyl acetate and observed with a JEM 1200EX (Nihon Denshi, Akishima, Tokyo, Japan) operating at 80 kV.

Quantification of Myosin Assembly—Myosin was assembled at 1.1 μM in 20 mM HEPES (pH 7.4), 1 mM DTT, 1 mM EGTA and various concentrations of KCl. To quantify the amount of assembled myosin, myosin filaments were sedimented by centrifugation at 100,000 × *g* for 40 min at 4°C, and the supernatant and precipitates were analyzed by SDS-PAGE. To discriminate amorphous aggregates from single myosin filaments, the myosin solution was centrifuged at 10,000 × *g* for 20 min at 4°C, and the supernatant was further centrifuged at 100,000 × *g* for 40 min at 4°C. The amount of assembled filaments was calculated by densitometry of the Coomassie-stained SDS-PAGE gel and processed with NIH image (ver 6.0), taking the molecular mass of myosin as 510 kDa.

Assembly of Myosin Rods—Rods were prepared according to the procedure described previously (*24*). Briefly, myosin was cleaved with α-chymotrypsin and applied to DEAE column chromatography on a column equilibrated with 20 mM Tris-HCl (pH 7.5), 20 mM K-pyrophosphate, 1 mM DTT, 1mM PMSF and 1 mM EDTA. The adsorbed rods were eluted with a linear gradient of K-pyrophosphate. For the sedimentation assay, rods were assembled at 1.0 μM in 20 mM HEPES (7.4), 1 mM DTT, 1 mM EGTA, and various concentrations of KCl. Then, the solution was centrifuged at 10,000 × *g* for 20 min at 4°C, and the supernatant and precipitates were analyzed by SDS-PAGE.

RESULTS

Sliding Movement of Ordinary and Dark Myosin—Ordinary myosin includes three kinds of light chains with masses of 16, 18, and 26 kDa on SDS-PAGE, and dark myosin includes two kinds of light chains with masses of 16 and 24 kDa (Fig. 1a), as described in the Introduction. Judging from the composition of the light chains on SDS-PAGE, the dark myosin in our myosin preparation was slightly contaminated with ordinary myosin, but the amount was less than 5% as calculated by densitometry.

When F-actin labeled with rhodamine-phalloidin was introduced into a flow cell, on the surface of which myosin was immobilized, smooth and continuous movement of the actin filaments was observed. The mean sliding velocity of the actin filament on ordinary myosin was 4.31 ± 1.67 μm/s at 50 mM KCl. This value agrees well with the value previously reported by Chaen *et al.* (*25*). The mean sliding velocity of actin filaments on dark myosin at 50 mM KCl was 2.82 ± 1.01 μm/s, which is two thirds that of ordinary myosin.

As no sliding movement on rabbit skeletal myosin occur at KCl concentrations over 60 mM (*17*), we examined this phenomenon of sliding movement on fish myosin by varying the KCl concentration with successive perfusion into the same flow cell. Sliding velocity was fairly constant at various concentration of KCl below the critical concentration (Fig. 1b). The critical concentrations of KCl for sliding movement were 75 mM for ordinary myosin and 65 mM for dark myosin (Fig. 1b). We repeated this experiment many times by increasing and then decreasing the KCl concentration by perfusion, and

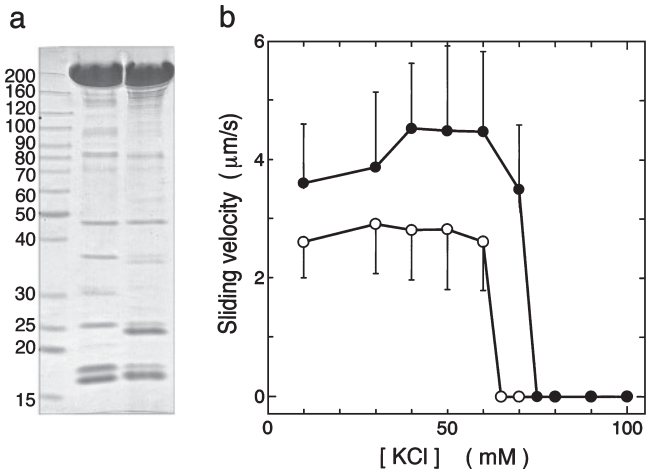

Fig. 1. **Sliding velocity of ordinary myosin and dark myosin at various concentrations of KCl.** (a) SDS-PAGE pattern of the myosin preparation used in this experiment. Lane 1, markers whose molecular masses are indicated on the left; lane 2, ordinary myosin; lane 3, dark myosin. Note that the light chains of ordinary myosin are 16, 18, and 26 kDa, and those of dark myosin as 16 and 24 kDa. (b) Sliding velocity obtained by the motility assay of ordinary and dark myosin. Open and closed circles indicate ordinary and dark myosin, respectively. Each data point represents the average of the sliding velocity of 30 actin filaments. The bar associated with each data point indicates standard deviation. Sliding movement could not be observed at KCl concentrations over 75 mM for ordinary myosin, and over 65 mM for dark myosin; below these critical KCl concentrations, the sliding velocity was almost constant.

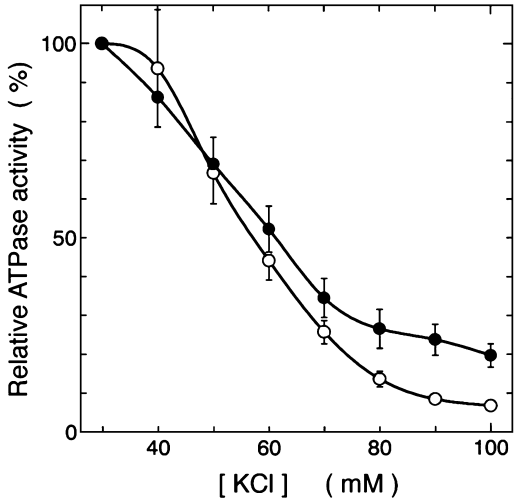

Fig. 2. **KCl dependency of the actin activated ATPase activity.** The ATPase activities at various concentrations of KCl were normalized using the value at 30 mM KCl as a standard. Concentrations of myosin and F-actin were 0.11 μM and 46.0 μM, respectively. The ATPase activity at 100% was 0.17 μmol P_i/min·mg for ordinary myosin, and 0.11 μmol P_i/min·mg for dark myosin. These data are averages of three independent experiments, and the bars indicate standard deviation. The data for ordinary and dark myosin are represented as open and closed circles, respectively.

confirmed that the value of the critical KCl concentration was reproducible. The actin filament could not bind to the myosin-coated surface above the critical concentration of KCl because of the very low affinity of myosin to F-actin. Our results suggest that the ordinary myosin has slightly higher affinity for F-actin than dark myosin.

We also examined the sliding movement on chicken fast myosin isolated from breast muscle to compare it with the sliding movement on carp ordinary myosin. The mean sliding velocity was 3.49 ± 1.05 μm/s at 50 mM KCl, slower than that of carp ordinary myosin, and the critical KCl concentration for movement was 65 mM. This indicates that carp ordinary myosin has a slightly higher affinity for F-actin than chicken fast myosin at KCl concentrations of 65 to 75 mM.

Actin Activated Mg^{2+}-ATPase—The actin-activated Mg^{2+}-ATPase activity of either type of myosin fell gradually with increasing concentrations of KCl (Fig. 2). The reduction in the activity was slightly steeper in case of dark myosin than ordinary myosin (Fig. 2). To obtain kinetic values of the activity, such as V_{max} and K_m, the ATPase activity was determined at varying the concentrations of F-actin and KCl (Fig. 3, a and b). The kinetic values of V_{max} and K_m obtained from the double reciprocal plots of these data are summarized in Table 1. V_{max} of the ATPase activity of ordinary myosin was about 1.6-times higher than that of dark myosin under motile conditions with KCl concentrations ranging from 30 to 60 mM. This value is very similar to the ratio of the sliding velocity of ordinary myosin to that of dark myosin: 1.5 times as described above (Fig. 1b). The K_m of ordinary myosin was lower than that of dark myosin at all KCl concentrations examined, suggesting that ordinary myosin has a higher affinity for F-actin than dark myosin.

Binding of the Myosin Head to the Actin Filament—The above results of the motility and ATPase assays indicate that ordinary myosin has a higher affinity for F-actin than dark myosin. To confirm this, we directly

Table 1. **Kinetic parameters of actin-activated Mg^{2+}-ATPase activity at various KCl concentrations.**

KCl (mM)	Ordinary myosin		Dark myosin	
	V_{max} (μmol P_i/min·mg)	K_m (μM)	V_{max} (μmol P_i/min·mg)	K_m (μM)
30	0.17	0.19	0.13	0.62
40	0.15	0.38	0.07	0.62
50	0.18	0.43	0.16	0.72
60	0.12	0.81	0.07	0.90
70	0.14	0.83	0.05	1.22
80	0.12	1.43	0.03	1.88

Values of V_{max} and K_m were calculated from double reciprocal plots of the data shown in Fig. 3 by the least mean squares method.

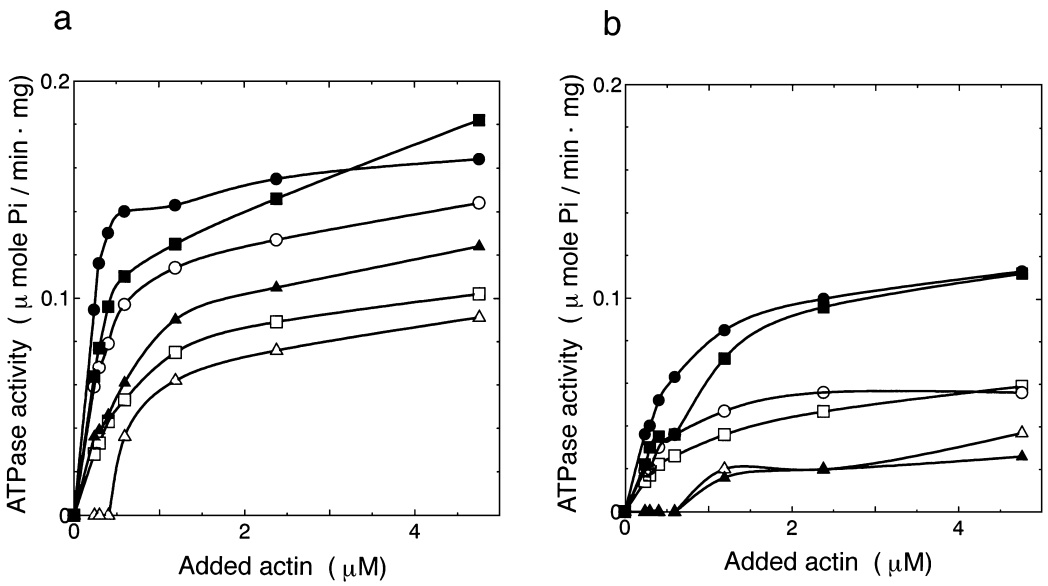

Fig. 3. **Actin activated ATPase activity at various KCl concentrations.** The ATPase activity was determined by varying the actin concentration at KCl concentrations of 30 mM (closed circles), 40 mM (open circles), 50 mM (closed squares), 60 mM (open squares), 70 mM (closed triangles), and 80 mM (open triangles). Ordinary myosin (a); dark myosin (b).

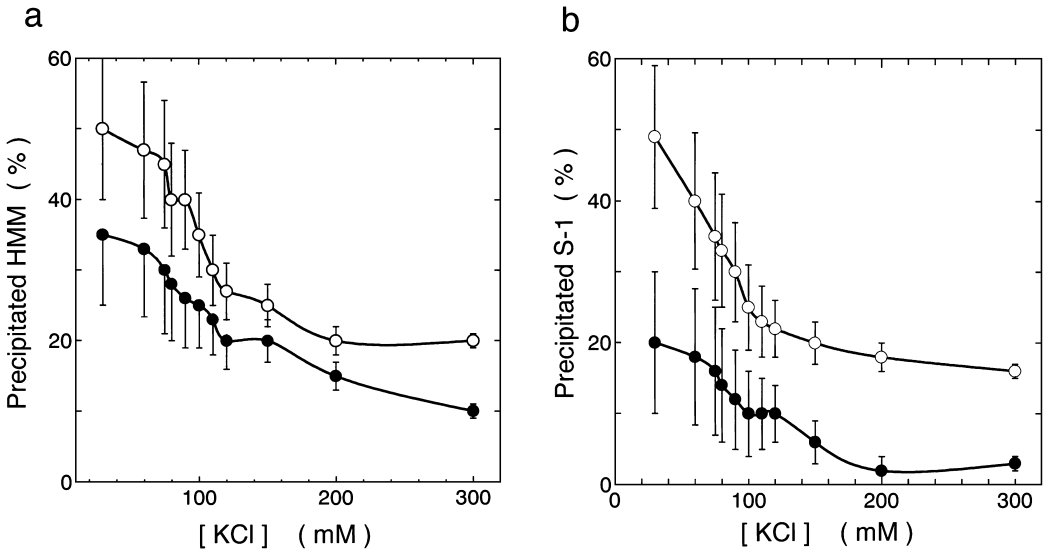

Fig. 4. **Binding assay of ordinary and dark myosin fragments to F-actin.** For the binding assay, myosin fragments HMM (a) or S-1 (b) were used. (a) Ordinary HMM had a higher affinity for F-actin than dark HMM. A mixture of HMM and F-actin (1.1 μM and 12 μM, respectively) in 20 mM HEPES (pH 7.5), 1 mM ATP, 2 mM $MgCl_2$, and 1 mM DTT was centrifuged. The precipitate and supernatant were analyzed by SDS-PAGE. (b) The binding experiment with S-1 was performed under the same buffer conditions as in (a). Concentrations of S-1 and F-actin were 1.1 μM and 12 μM, respectively. The data are averages of three independent experiments. Open and closed circles represent ordinary and dark myosin fragments, respectively.

quantified the binding of myosin heads to F-actin. The soluble fragment of myosin, HMM or S-1, was mixed with F-actin in the presence of ATP, and the mixture was centrifuged to quantify the amount of fragments that co-precipitated with F-actin. The amounts of ordinary HMM and also S-1 bound to F-actin were larger than those of dark myosin at the KCl concentrations examined (Fig. 4, a and b). The amount of HMM bound to F-actin was greater than that of S-1 in both the ordinary and dark myosin fragments. This might be due to the fact that HMM has two heads while S-1 has only one head to interact with F-actin. Upon increasing the concentration of KCl, dark HMM was released from F-actin at the lower concentration of KCl than that of ordinary HMM (Fig. 4a). Similarly, dark S-1 was dissociated from F-actin at lower concentrations of KCl than ordinary S-1 (Fig. 4b). These observations on the binding of HMM and S-1 to F-actin suggest that ordinary myosin has a higher affinity for F-actin than dark myosin.

Shape of Self Assembled Myosin—Myosin from ordinary and dark muscle assembles into filaments under physiological conditions. By electron microscopy, great

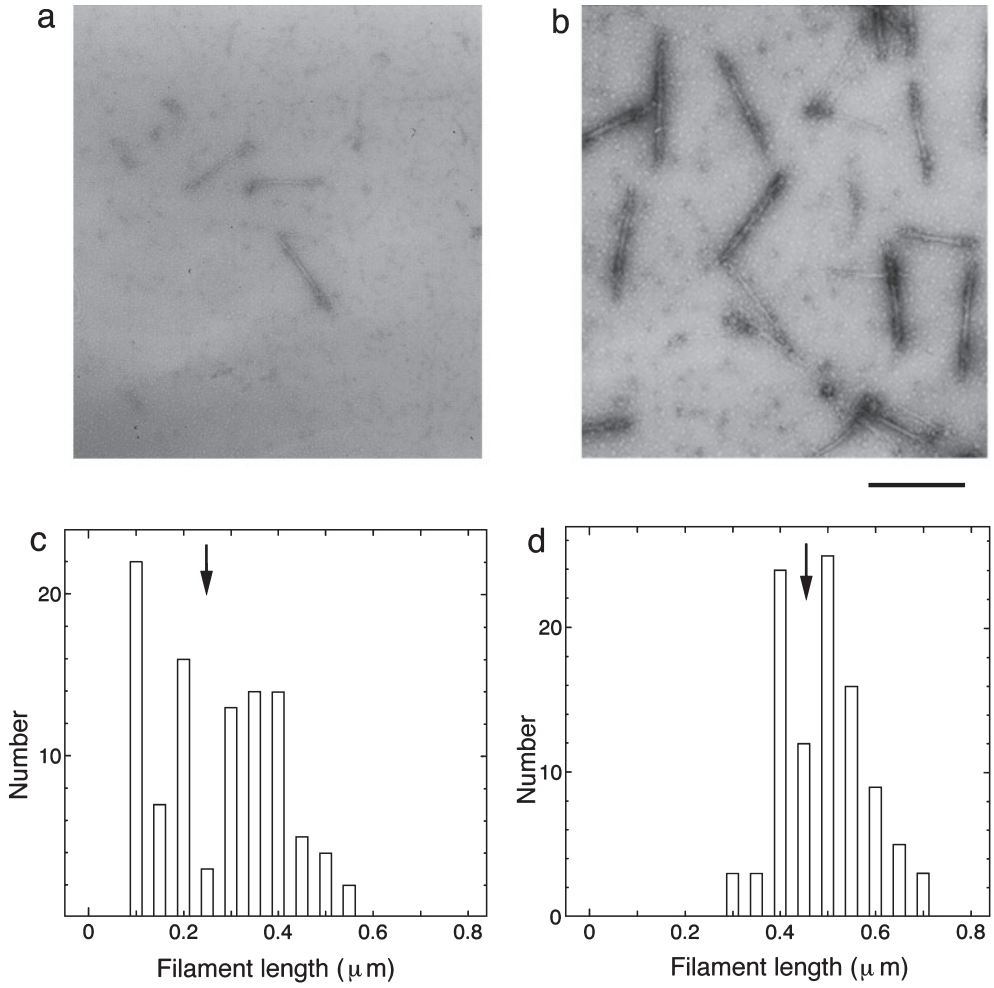

Fig. 5. **Assembly of ordinary and dark myosin.** Electron micrographs of ordinary (a) and dark myosin (b) assembled at 150 mM KCl are shown. The bar under (b) represents 0.5 μm. Histograms of the length of 100 individual myosin filaments of ordinary (c) and dark myosin (d). Arrows show the average lengths. The mean lengths of ordinary and dark myosin filaments were 0.25 and 0.47 μm, respectively. Self assembly of ordinary myosin was poorer than that of dark myosin.

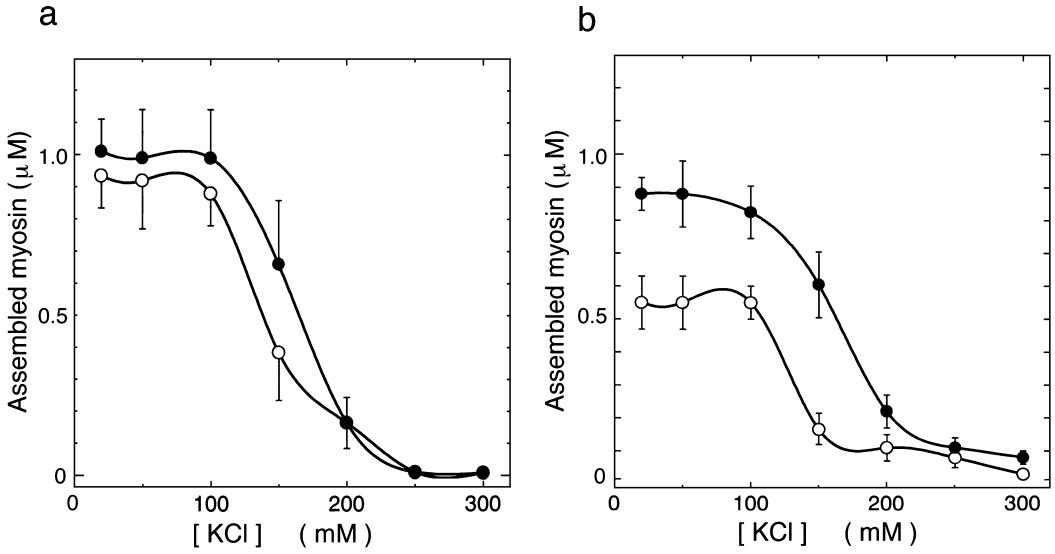

Fig. 6. **Centrifugation assay to quantify single myosin filaments.** (a) Centrifugation assay at 100,000 × *g*. (b) Stepwise centrifugation assay. A myosin solution was centrifuged at 10,000 × *g* and then the supernatant was further centrifuged at 100,000 × *g* at various concentrations of KCl. There were differences in the assembly profile between stepwise centrifugation (b) and one step centrifugation at high gravity (a). Each data point shows the average of three independent experiments. Open and closed circles indicate ordinary and dark myosin, respectively.

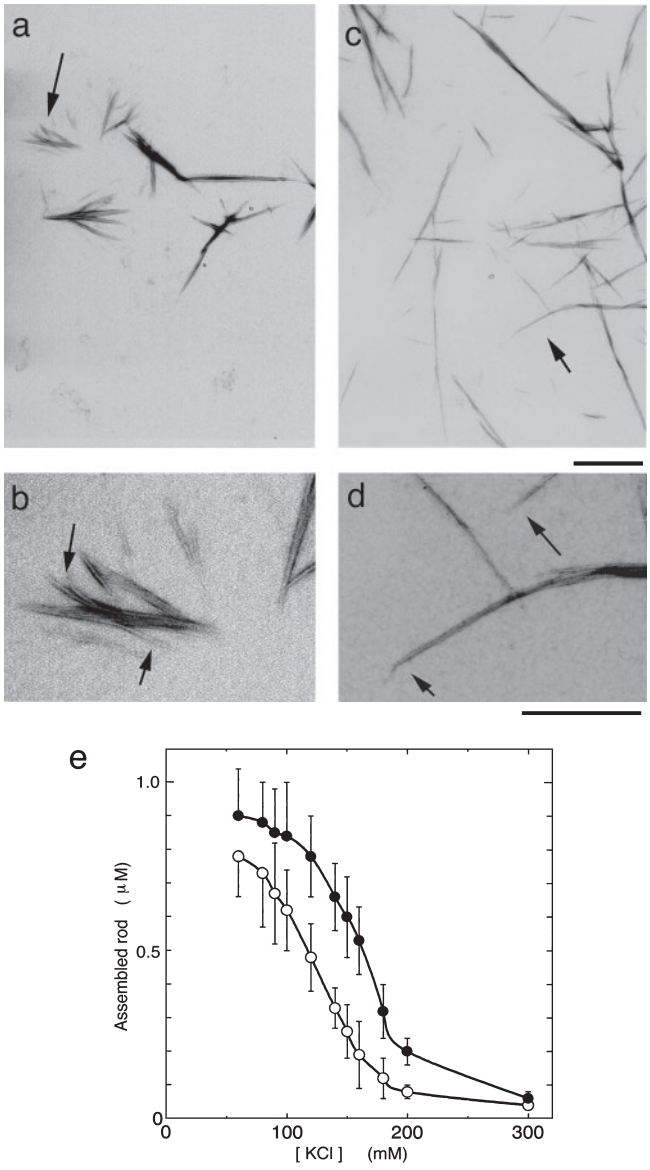

Fig. 7. **Electron micrographs of ordinary and dark myosin rod filaments and quantification of assembly.** (a) and (b) Electron micrograph of ordinary rods (a) at low and (b) high magnification. The edge of the filament pointed out by the arrow in (a) is shown magnified in (b). Note that the edges of the filaments of ordinary rods are usually loose and splayed as pointed out by the arrows. (c) and (d) Electron micrograph of dark myosin rods at low magnification (c) and high magnification (d). The edge of the dark myosin rod is tapered, as is clearly visible in (d) as pointed out by arrows. Magnifications in (a) and (c) are the same (bar indicates 4 μm), and those of (b) and (d) are the same (bar indicates 2 μm). (e) Amount of assembled rods at various KCl concentrations determined by centrifugation. Ordinary (open circles) and dark rods (closed circle). A solution of rods was centrifuged at 10,000 × *g*, and the supernatant and precipitate were analyzed to estimate assembled myosin rods. The data are averages of three independent experiments. The bars indicate standard deviations.

differences in the shape of the filaments can be seen between these types of myosin. Ordinary myosin forms shorter and thinner filaments than dark myosin, as shown in Fig. 5a. We often observed that short myosin filaments composed of small numbers of myosin molecules were associated with each other to form side-by-side, or sometimes amorphous, aggregates several micrometers in length (data not shown). The lengths of 100 individual filaments of ordinary myosin were distributed broadly as shown in Fig. 5c. The mean length of a filament was 0.25 ± 0.13 μm.

The filaments composed of dark myosin clearly showed a bipolar shape, and were dispersed individually as shown in Fig. 5b. In contrast to the assembly of ordinary myosin, when the same amount of myosin was mounted on a mesh, many filaments of dark myosin could be seen under an electron microscope. The distribution of the lengths of 100 individual filaments showed a narrow single peak with a mean length of 0.47 ± 0.09 μm (Fig. 5d).

Quantification of Myosin Assembly by Stepwise Centrifugation—From the above electron microscopic observations, the amount of ordinary myosin assembled into filaments was expected to be apparently lower than that of dark myosin. But from the centrifugation assay at 100,000 × *g*, the amount of assembled myosin did not differ significantly between ordinary and dark myosin (Fig. 6a). This would be due to the fact that both aggregated material and single filaments are precipitated under these conditions. To quantify the amount of single myosin filaments more accurately, the myosin solution was centrifuged once at low speed (10,000 × *g*) to sediment aggregates, and then the supernatant was further centrifuged at high speed (100,000 × *g*) to sediment single filaments at 150 mM KCl. At the lower speed, most of the single myosin filaments did not precipitate (data not shown). As shown in Fig. 6b, the amounts of myosin filaments of ordinary and dark myosin were clearly different by the stepwise assay as compared with the results of the one-step assay shown in Fig. 6a.

The amounts of aggregates, single filaments and monomeric myosin were estimated from the data of the high speed and low speed centrifugations as shown in Fig. 6, a and b. Monomers, aggregates, and single filaments comprised 64, 22, and 14% of ordinary myosin, respectively, and 41, 4, and 55% of dark myosin, respectively. The amount of single filaments of dark myosin was 3.9 times greater than that of ordinary myosin. Thus, these results agree well with the observations by electron microscopy.

Mammalian or avian skeletal muscle myosin prepared by the conventional method of repeated assembly and disassembly usually contains several myosin-binding proteins, such as C-protein or H-protein (*26–28*). Most of these myosin-binding proteins can be removed by purification by DEAE column chromatography. To remove these myosin binding proteins, dark myosin and ordinary myosin were further purified by DEAE column chromatography. By electron microscopy, the filament structures of the column purified myosin were very similar to those before chromatography (data not shown). Thus the poor assembly of ordinary myosin appears to be an inherent property.

Assembly of Myosin Rods—As described above, ordinary myosin, but not dark myosin, tends to form aggregates. To clarify whether the poorer assembly of ordinary myosin is derived from its partial denaturation or its true nature, we prepared rods and examined their assembly. As shown in Fig. 7a, ordinary rods appeared to assemble

into short filaments and also side-by-side aggregates, and the edges of the filaments were often loose or splayed (Fig. 7b). On the other hand, dark rods assembled into long, tight filamentous structures, and their ends were tapered (Fig. 7, c and d). Abundant filaments of dark rods dispersed on a mesh were also observed under the electron microscope, as observed in the case of dark myosin.

To compare the amount of assembled filaments of ordinary and dark rods, we performed the centrifugation assay at low speed, 10,000 × *g*. When the solution of rods was centrifuged at 10,000 × *g*, most of the assembled rods sedimented, since the size of the assembled rods (Fig. 7, a and c) was much greater than that of single myosin filaments (Fig. 5, a and b). The amount of assembled ordinary rods was about half that of assembled dark rods at 150 mM KCl, and the profile of rod assembly shown in Fig. 7e agrees well with that of myosin shown in Fig. 6b. Thus we confirmed that the poorer assembly of ordinary myosin than dark myosin originates from the inherent nature of ordinary myosin.

DISCUSSION

We have here described the mechanochemical properties of carp ordinary and dark myosin. The V_{max} of the ATPase activity of ordinary myosin is 1.6 times higher and the sliding velocity 1.5 times faster than those of dark myosin. The ATPase activities of myofibrils from carp and sardine ordinary muscle are reported to be 1.4 and 1.5 times higher than those of dark muscle, respectively (*29*, *30*). Since muscle has a highly organized structure, the ATPase activity of the myofibril will correspond to V_{max}. From our preliminary examination, the values of the ratio of V_{max} and sliding velocity of ordinary to dark myosin from great amberjack, horse mackerel, and Japanese sea perch are all between 1.3 and 1.8 times (unpublished results). Thus, the sliding velocity and V_{max} of the ATPase activity of ordinary myosin seems to be only slightly higher than that of dark myosin.

There are reports, however, that the ATPase activities of ordinary myosin of mackerel (*5*) and yellowtail (*6*) are 5.8 and 3.2 times higher than those of dark myosin, respectively. This discrepancy may come from differences in the experimental conditions. The point is that they measure the activities at a weight ratio of actin to myosin of 1 to 1. Under these experimental conditions, the ratio of the ATPase activity of ordinary myosin to that of dark myosin can become higher than the ratio of V_{max} if the K_m of dark myosin is much larger than that of ordinary myosin, as in the case of carp myosin (Table 1).

In contrast to fish myosin, mammalian fast myosin is reported to have a 3 to 10 times higher activity. The actin-activated ATPase activity of rabbit fast (white) myosin is 4 times greater than that of slow (red) myosin (*2*, *31*). Human fast myosin type IIA and IIB show ATPase activities 3 and 10 times higher than slow myosin type I, respectively (*32*, *33*).

The sliding velocity of rat fast myosin obtained from a single muscle fiber is 4 times faster than that of slow myosin (*34*, *35*). The maximum shortening velocity of chicken fast muscle is 10 times faster than that of slow muscle (*36*). Interestingly, the sliding velocity of carp ordinary myosin is 4.31 μm/s (Fig. 1b), which is very similar to that of mammalian or avian fast myosin; for instance, 5.4 μm/s for rabbit (*17*), 5.83 μm/s for rat (*34*), or 3.49 μm/s for chicken fast myosin as described in Results. On the contrary, the sliding velocity of dark myosin is 2.82 μm/s (Fig. 1b), which is twice that of mammalian slow muscle myosin, for instance, 1.43 μm/s for rat slow myosin (*34*).

Fish uses only dark muscle for slow cruising, whereas ordinary muscle is used only for sudden movements such as escape from an enemy or catching food (*5*–*7*). The fact that the sliding velocity and ATPase activity of dark myosin in fish are much higher than those of mammalian slow muscle myosin would account for this physiological role of dark muscle in the life style of fish.

We have also found that the filament formation of ordinary myosin is about one- fourth that of dark myosin under physiological conditions of 150 mM KCl (Fig. 6b). Ordinary myosin assembles poorly at a concentration of 1.1 μM (Fig. 6), which is two orders lower than the myosin concentration in living muscle (*37*). On the other hand, mammalian fast myosin assembles almost fully into filaments *in vitro* at 150 mM KCl. This indicates that the critical concentration of ordinary myosin is much higher than that of mammalian fast myosin, and it may be a little lower than 1 μM, because most ordinary myosin was monomeric under our experimental conditions. However in muscle, the myosin concentration is around two orders of magnitude higher than this critical concentration, so that almost all ordinary myosin molecules are in the filamentous state in muscle.

Compared with the synthesized myosin filament, native thick filaments isolated directly from carp ordinary muscle are much more stable at 150 mM KCl (unpublished result). This suggests that a stabilizing protein may be present in ordinary muscle, and that it plays an important role in the organization of myosin assembly in muscle. In mammalian skeletal muscle, there are some myosin binding proteins, such as C-protein and M-protein (*26*, *38*, *39*). Both proteins bind to myosin to maintain the localization of the thick filament at the center of the sarcomere, but do not promote myosin assembly. And further, C-protein preparations isolated from carp ordinary or dark muscle did not stimulate assembly of ordinary or dark myosin (unpublished result). Based on these facts, the myosin-binding proteins thus far isolated from mammalian skeletal muscle, including C-protein or M-protein, are not candidates for the myosin stabilizing factor in this case. Poor assembly of ordinary myosin might reflect some characteristic functions, and the possibility of a putative stabilizing factor in carp ordinary myosin filament should be investigated in the near future.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and a grant from the Uehara Memorial Foundation. We thank our colleagues at Mie University for important advice and discussion. Especially we thank Dr. T. Nakayama for encouragement, and Mr. F. Yano for technical help at the beginning of this experiment.

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