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Recombinant motor domain constructs of *Chara corallina* myosin display fast motility and high ATPase activity

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Abstract

The mechanism and structural features that are responsible for the fast motility of *Chara corallina* myosin (CCM) have not been elucidated, so far. The low yields of native CCM that can be purified to homogeneity were the major reason for this. Here, we describe the expression of recombinant CCM motor domains, which support the fast movement of actin filaments in an in vitro motility assay. A CCM motor domain without light chain binding site moved actin filaments at a velocity of 8.8 μm/s at 30 °C and a CCM motor domain with an artificial lever arm consisting of two α-actinin repeats moved actin filaments at 16.2 μm/s. Both constructs displayed high actin-activated ATPase activities (~500 Pi/s/head), which is indicative of a very fast hydrolysis step. Our results provide an excellent system to dissect the specific structural and functional features that distinguish the myosin responsible for fast cytoplasmic streaming.

Keywords: Myosin; Motor protein; Characean; Sliding movement; Cytoplasmic streaming; ATPase

Cytoplasmic streaming in characean algal cells is extremely fast (about 70 μm/s at 24 °C). It has been suggested that this fast cytoplasmic streaming is generated by the sliding movement of myosin along the fixed actin bundles in the cells [1–3]. Myosin from *Chara corallina* moved actin filaments in vitro at the maximal velocity of ~50 μm/s [4,5]. The velocity is about 10 times faster than that of skeletal muscle myosin and it is the highest measured for a molecular motor protein, so far. Myosin converts chemical energy liberated by ATP hydrolysis into mechanical motion. Because *Chara corallina* myosin (CCM) is unique in its high actin sliding velocity, critical information about the chemo-mechanical energy conversion would be obtained by investigating fast CCM-dependent actin movement. However, the yields of CCM are very low when the native protein is purified from *Chara* cells. Therefore, it has been difficult to measure the detailed kinetics of native CCM. In addition, the activity of CCM is impaired after multiple purification steps, so that in vitro actin sliding velocity of CCM purified to homogeneity is only 1/4–1/2 of that measured with crude extracts of *Chara* cells [4,6]. Therefore, expression of functional recombinant CCM is needed to obtain reliable biochemical and structural information about the CCM motor domain.

Recently, the cDNA of the CCM myosin heavy chain was cloned [7,8]. The primary sequence deduced from the cDNA predicts a motor domain that belongs to class XI myosin, a neck domain comprising six tandem repeats of IQ motifs and serving as binding sites for six calmodulin-like myosin light chains, an α-helical coiled-coil domain supporting dimer formation, and a globular tail domain (Fig. 1, Native *Chara* Myosin). Overall, this sequence derived structure agrees well with that of native CCM observed by electron microscopy [9].

The functional expression of myosins requires co-expression of the light and heavy chains. It has been reported that calmodulin functions as light chains for

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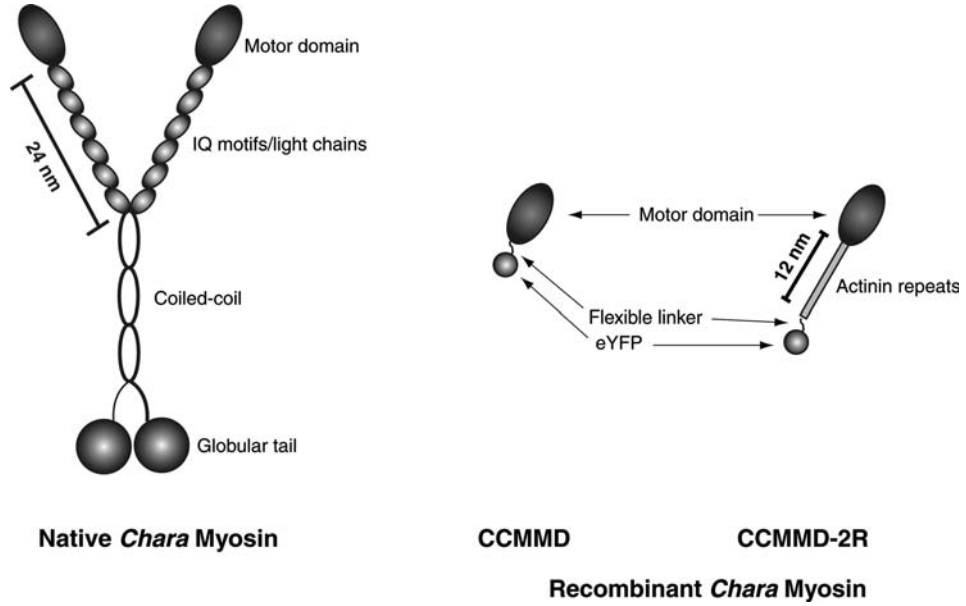

Fig. 1. Schematic diagrams showing native *Chara* myosin and the recombinant *Chara* myosin constructs used in this study. The domain structure of native CCM has been deduced from its amino acid sequence. Native Chara myosin has a neck region with six IQ motifs. Thus, it is supposed that six calmodulin-like light chains bind to each heavy chain. The length of the six IQ motifs is estimated to be 24 nm. According to the lever arm model, the neck region serves as amplifier of smaller conformational changes in the motor domain. CCMMD is composed of the globular head of *chara* myosin followed by a flexible linker and eYFP. CCMMD-2R has an elongated rigid domain consisting of two α-actinin repeats inserted between the globular head and the flexible linker of CCMMD. The combined α-actinin repeats are 12 nm long and they are expected to function as artificial lever arm.

many unconventional myosins [10–17]. However, the CCM heavy chain did not associate with *Chara* calmodulin when both polypeptides were co-expressed using the baculovirus–insect cell system (Kashiyama and Awata, unpublished observation). Previously, we expressed a chimeric myosin comprising the motor domain of CCM and the neck and tail domains of *Dictyostelium* myosin II [18]. This construct has two IQ motifs, to which the regulatory and essential light chains of *Dictyostelium* myosin II bind. The length of the lever arm of this chimeric *Chara* myosin was one-third of that of the native *Chara* myosin [7,8]. According to the lever arm theory, actin sliding velocity should be proportional to the length of the lever arm [19,20]. Therefore, the velocity produced by the chimeric myosin was expected to be one-third of that of native CCM, ~17 μm/s. However, contrary to expectation, the observed actin sliding velocity was only 3.2 μm/s [18].

There might be several reasons for this lower velocity of the chimeric myosin. The most likely reason is that the light chains and/or light chain binding sites of *Dictyostelium* myosin II interfere with conformational changes in the CCM motor domain. In fact, it has been shown that the N-terminus of the motor domain interacts with the essential light chain [21,22]. It has also been reported that the actin sliding velocity of a chimeric myosin comprising a skeletal muscle myosin motor domain and the neck domain (light chain binding site and light chains) of smooth muscle myosin was only 1/40 of that of skeletal myosin [23]. Therefore, we generated two constructs that were designed to avoid the interference by light chains from a different kind of myosin. One construct corresponds to the CCM motor domain (CCMMD) fused to enhanced yellow fluorescent protein (eYFP) by a flexible joint. This CCMMD construct does not have light chain binding sites and light chains (Fig. 1, CCMMD). The other to which we refer as CCMMD-2R consists of the CCMMD and two triple coiled-coil repeats of α-actinin connected to eYFP via a flexible linker (Fig. 1, CCMMD-2R). The rigid α-actinin repeats have been shown to function as lever arm when fused to the motor domain of *Dictyostelium* myosin II [20,24,25]. Both constructs had much higher motile and ATPase activity than the chimeric CCM constructs produced previously. Our results provide definite evidence that the cloned cDNA encodes the myosin responsible for fast cytoplasmic streaming of *Chara corallina*. The successful expression of these CCM constructs in this study opens the possibility to unravel the mechanistic and structural features responsible for their high motile activity.

Materials and methods

Reagents. Restriction enzymes and modifying enzymes were purchased from New England Biolabs (Beverly, MA). EDTA, EGTA, DTT, and ATP were purchased from Wako Chemicals (Osaka, Japan). Protease inhibitors and phalloidin were purchased from Sigma (St. Louis, MO).

Plasmid construction and protein expression. The expression vector for the production of CCMMD-2R was generated as follows. Plasmid

pTIKL CCM730/Dd II neck and tail, which encodes Glu4–Gln730 of the CCM heavy chain and Leu751-A2116 of *Dictyostelium* myosin II heavy chain [18], was mutated by site-directed mutagenesis using the ExSite PCR-Based Site-Directed Mutagenesis Kit (Stratagene, CA). The sequences of the oligonucleotides used to create the mutations were 5′-CTAATGTTTTAAATGAAGCTGCTTCTCGAGTTAGCT CGTATTGAAGAAGCTCGTG-3′ and 5′-ATCTTAAGGTATCT AAAATAGCCATTTGACCGGCGCGCAGG-3′. The resultant plasmid, pTIKL CCM746/DdII neck and tail, encoded Glu4–Ala746 of the CCM heavy chain. This plasmid was cut with *Xho*I and *Sac*I and ligated with a *Xho*I–*Sac*I fragment from pM790-2R-eYFP (S. Zimmermann, D.J. Manstein, unpublished work). The resultant construct encodes CCM heavy chain residues 4–746 followed by Gln266–Asp503 of *Dictyostelium* α-actinin, a flexible linker (GSGGSGGSGGSG), eYFP, and a $(His)_8$-tag.

PTIKL CCMMD was made as follows. A *Xho*I site was created upstream of the flexible linker of pM790 2Re YFP by site-directed mutagenesis using the ExSite PCR-Based Site-Directed Mutagenesis Kit. The sequences of the oligonucleotides used to create the mutations were 5′-GATCTAGAATCTCGATCTTCAATCTTTTGG-3′ and 5′-TCGA GCAGGATCAGGAGGATCAGGAGG-3′. The resultant DNA encodes the flexible linker, eYFP, and $(His)_8$-tag. This construct was cut with *Xho*I and *Sac*I and ligated with *Xho*I–*Sac*I digested pTIKL CCM 746/DdII neck and tail. The resulting construct encodes CCM heavy chain residue 4–746 followed by a flexible linker (GSGGSGGSGGSG), eYFP, and a $(His)_8$-tag. Residue 746 of CCM corresponds to residue 766 of *Dictyostelium* myosin II. It was shown previously that loss of the peptide between 754 and 761 of *Dictyostelium* myosin II leads to decreased thermal stability and altered kinetic behavior, suggesting that this region is important for proper motor protein function [26,27].

The resultant pTIKL CCMMD and pTIKL CCMMD-2R were separately electroplated into *Dictyostelium* Ax2 cells and transformants were selected in the presence of 30 μg/ml G418 in the HL5 medium containing 60 μg/ml each of penicillin and streptomycin.

Protein purification. CCMMD and CCMMD-2R were purified as described [28]. Rabbit skeletal muscle actin was prepared using the method of Spudich and Watt [29]. Protein concentration was determined using the CoomassiePlus protein assay reagent (PIERCE, IL) for CCMMD and CCMMD-2R with *Dictyostelium* S1 as the standard. The concentrations of *Dictyostelium* S1 and actin were determined spectrophotometrically using extinction coefficients of 0.80 cm^2/mg at 280 nm for S1 [30] and 0.62 cm^2/mg at 290 nm for actin [31].

In vitro motility assay. An antibody-based version of the in vitro sliding filament assay was used, corresponding to a modified version of the method described by Reck-Peterson et al. [32]. Briefly, a flow chamber (5×10 mm, about 7 μl of chamber volume) was prepared using a nitrocellulose coated coverslip and a glass slide, as described by Kron et al. [33]. Protein G (0.5 mg/ml in Hepes, pH 7.4, Zymed Laboratories, CA) was adsorbed to the surface of the flow chamber for 30 min at room temperature. The chamber was washed with 4×100 μl HBS (10 mM Hepes–KOH, pH 7.4, 150 mM NaCl). Next, anti-GFP monoclonal antibody (0.2 mg/ml in HBS, Sigma, MO, Cat. No. G6539) was introduced into the chamber and left for 3 h at 4 °C. After antibody adsorption, the chamber was washed with 4×100 μl HBS. Then, CCMMD or CCMMD-2R (0.1–0.2 mg/ml in HBS) was added to the chamber and left for 1–3 h at 4 °C. Before the introduction of F-actin labeled with rhodamine–phalloidin, the myosin coated flow chamber was washed with unlabeled F-actin and Mg^{2+}-ATP in order to block residual denatured myosin. The assay buffer used in the in vitro motility assay contained 25 mM Hepes, pH 7.4, 25 mM KCl, 4 mM $MgCl_2$, 1 mM EGTA, 2 mM ATP, 10 mM DTT, 12.8 mM glucose, 120 μg/ml of glucose oxidase, and 20 μg/ml catalase. The in vitro motility assay was performed at 30 or at 22 °C. Average sliding velocities were determined by measuring the displacements of actin filaments that were smoothly moving for distances greater than 10 μm.

ATPase assays. Steady-state ATPase activities were determined by measuring released phosphate using the method of Kodama et al. [34] under the modified conditions described by Ruppel et al. [35]. We added 1 mg/ml BSA in the reaction mixture to prevent adsorption of motor proteins to plastic tubus and to stabilize motor proteins. The reaction mixtures for the assay of the basal Mg^{2+}-ATPase activity contained 25 mM Hepes (pH 7.4), 25 mM KCl, 4 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 1 mg/ml BSA (Sigma, MO, Cat. No. A0281), and 3 μg/ml CCMMD or CCMMD-2R. The reaction mixtures for the assay of actin-activated Mg^{2+}-ATPase activity contained 25 mM Hepes (pH 7.4), 25 mM KCl, 4 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 1 mg/ml BSA, 0.125–4 mg/ml F-actin, and 0.1–0.5 μg/ml CCMMD or CCMMD-2R. The reactions were initiated by the addition of ATP at 30 °C. The ATPase reaction was stopped by adding perchloric acid after 0, 10, 20, and 30 min of incubation for the basal Mg^{2+}-ATPase activity and 0, 1, 2, and 3 min of incubation for the actin-activated Mg^{2+}-ATPase activity. A straight line was drawn by linear regression. Under this experimental condition, ATP hydrolysis rate was constant among four time points, showing that the high ATPase reaction was successfully measured.

Cosedimentation assays. CCMMD (0.5 μM) and phalloidin–actin (2 μM) were mixed in the cosedimentation buffer (25 mM Hepes, 100 mM KCl, 4 mM $MgCl_2$, and 1 mM DTT). After 10 min incubation, they were centrifuged at 200,000*g* for 10 min at 22 °C with (3 mM) or without ATP.

Results and discussion

Constructs

Fig. 1 shows the two recombinant CCM motor domain constructs used in this study. Construct CCMMD contains residues 4–746 of the CCM heavy chain, a flexible linker (GSGGSGGSGGSG), eYFP, and a histidine-tag. The CCMMD-2R construct contains the same domains and structural elements. However, while CCMMD lacks a lever arm region, CCMMD-2R has two α-actinin repeats inserted between the motor domain and the flexible linker. The rigid α-actinin repeats have been shown to function as lever arms when fused to the *Dictyostelium* myosin II motor domain [20,24,25]. Both constructs were over-produced in *Dictyostelium* wild type cells (Ax2) and purified using the protocols developed for similar *Dictyostelium* myosin II constructs [28]. Yields and purity of both constructs were about 0.1 mg per 10 g of wet cells and >95%, respectively (Fig. 2).

Motility

To investigate the motile activity of CCMMD and CCMMD-2R, we used an antibody-based version of the in vitro sliding filament assay [32,36]. The constructs were fixed to a glass surface by anti-GFP monoclonal antibody (see also, Materials and methods). Both CCMMD and CCMMD-2R supported continuous movement of actin filaments.

CCMMD moved actin filaments at a velocity of 8.8 ± 0.5 μm/s at 30 °C ($n = 81$, Fig. 3) and 6.2 ± 0.5 μm/s at 22 °C ($n = 30$). This velocity is unusually fast for a construct without proper lever arm.

Because the folded conformation of CCMMD deduced by its amino acid sequence is the same as that of

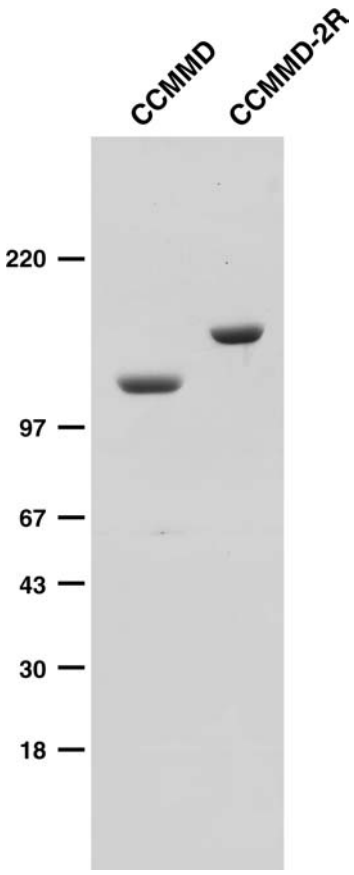

Fig. 2. SDS–PAGE of purified CCMMD and CCMMD-2R. The purity of CCMMD and CCMMD-2R was analyzed by SDS–PAGE using 12% gels. Note that the purity of the individual motor domain constructs was >95%.

Dictyostelium myosin II MD, we inferred the lever arm length of CCMMD from the crystal structure of *Dictyostelium* myosin II MD (Fig. 4, MD). Structural and molecular genetic studies have shown that the converter domain rotated around the switch-2 helix, which is also referred to as relay helix [24,37–40]. The fulcrum for the converter domain is at the distal end of the SH1 helix [37]. The residual lever arm length of the MD stemming from this rotation is 3 nm (Fig. 4, MD). Between CCM and the surface, there is a flexible linker, eYFP, anti-GFP antibody, and protein G, and it is not obvious where the other fulcrum point might be for the lever arm action, or whether eYFP, antibody, or protein G might contribute to the lever arm length. In the following argument, however, we assume that the other fulcrum point is at the flexible linker between CCM and eYFP (Fig. 1). Based on this assumption, the length of the lever arm of CCMMD is 3 nm, which is about 1/9 of that of the native CCM (27 nm, the sum of 3 nm for the converter region rotation and 24 nm for the light chain binding sites) [7,8]. If the sliding velocity is proportional to the lever arm length as proposed by the lever arm theory [19,20], the expected velocity of CCMMD is 1/9 of that of the native CCM. The velocity of cytoplasmic streaming in *Chara* cells at 30 and 22 °C is approximately 100 and 70 μm/s. Therefore, the expected velocity of CCMMD at 30 and 22 °C is around 11 and 7.7 μm/s, respectively. These values are similar to the velocity that was actually measured with CCMMD (8.8 μm/s at 30 °C, 6.2 μm/s at 22 °C). This indicates that CCMMD is fully functional molecular motor.

CCMMD-2R moved actin filaments at 16.2 ± 1.1 μm/s at 30 °C ($n = 90$, Fig. 3) and 12.2 ± 1.8 μm/s at 22 °C

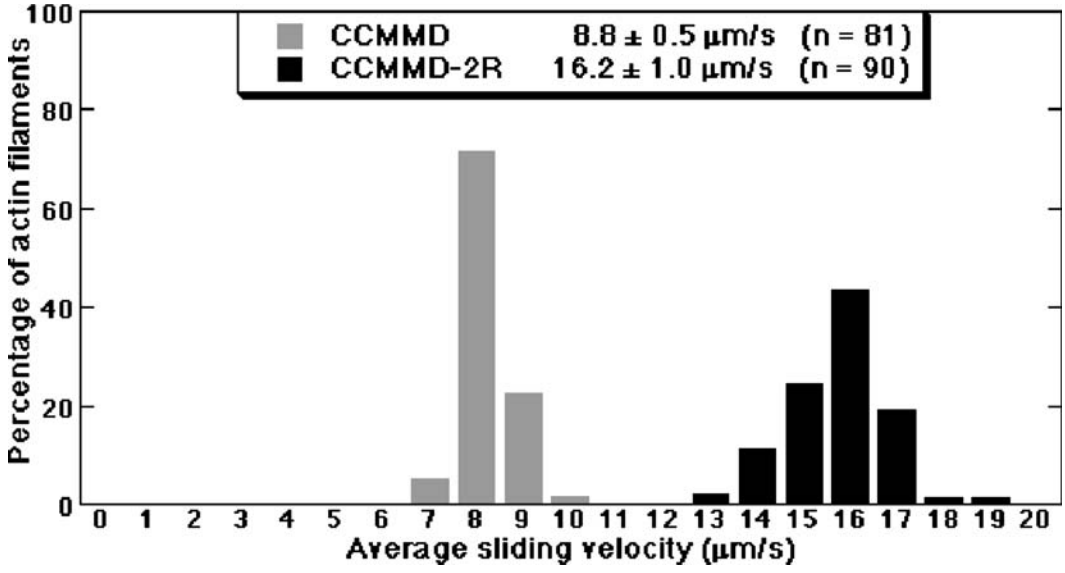

Fig. 3. Histogram of the distribution of velocities of actin filaments sliding in the in vitro motility assay over CCMMD or CCMMD-2R surfaces. Only filaments with a minimum run length of 10 μm were scored. Measurements for each construct were made with 80–90 actin filaments at 30 °C. Average actin sliding velocity of CCMMD and CCMMD-2R was 8.8 ± 0.5 and 16.2 ± 1.0 μm/s, respectively (mean ± SD).

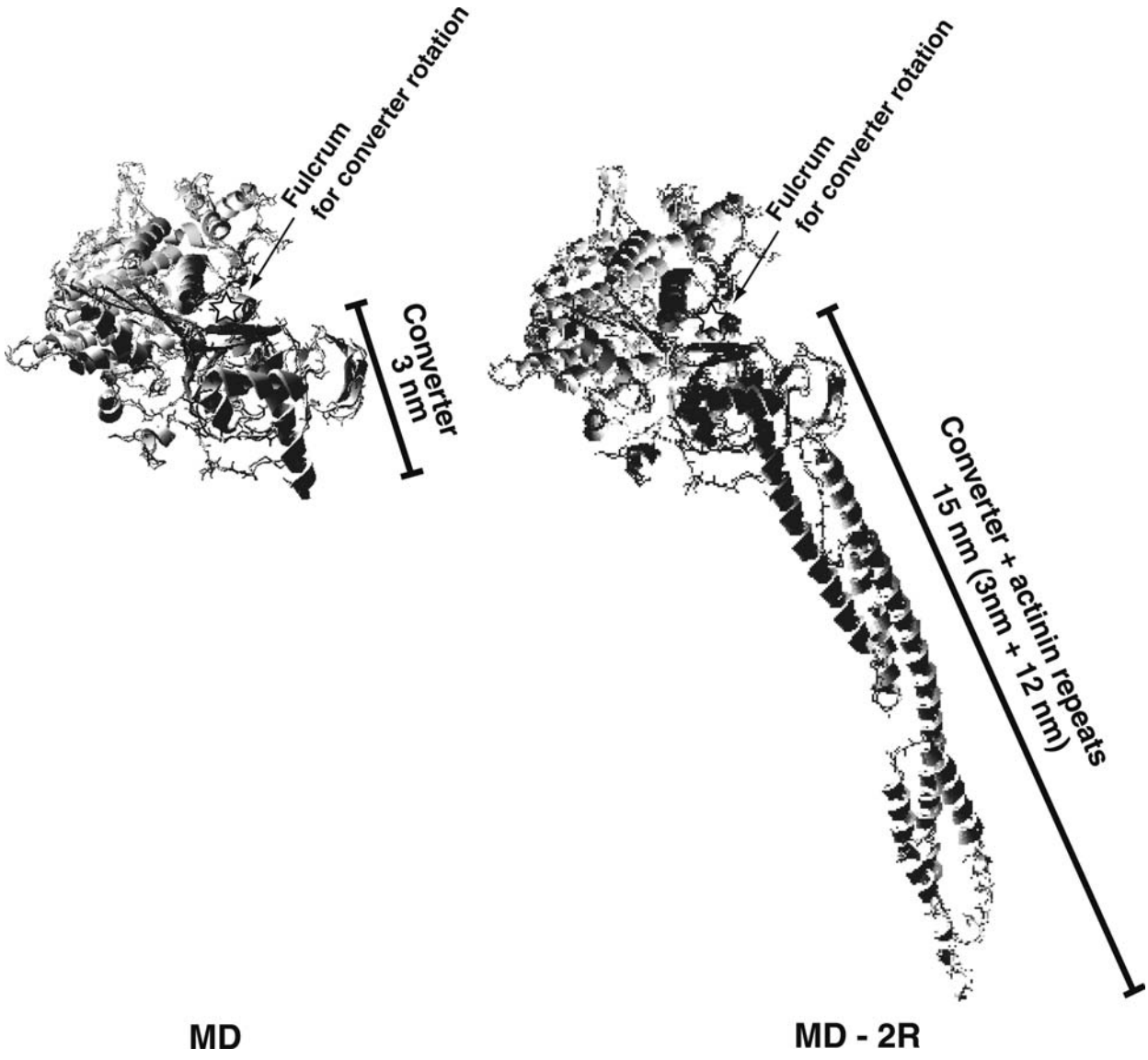

Fig. 4. Lever arm length of MD and MD-2R. MD, crystal structure of *Dictyostelium* myosin II MD (residues 2–766). Residue 766 of *Dictyostelium* myosin II corresponds to residue 746 of *chara* myosin. The fulcrum for the converter rotation is at the distal end of the SH1 helix, shown by star. The substantial lever arm length of MD by this rotation is 3 nm. MD-2R, crystal structure of *Dictyostelium* myosin II MD with the rigid α-actinin repeats. The substantial lever arm length of MD-2R by the converter rotation is 14 nm.

($n = 60$). Our estimate of the lever arm length of CCMMD-2R is 15 nm, which is the sum of 3 nm for the converter region rotation and 12 nm for the rigid α-actinin repeats (Fig. 4, MD-2R) [25]. Therefore, the lever arm length of CCMMD-2R is about five times longer than that of CCMMD, and the velocity of CCMMD-2R is about two times faster than that of CCMMD. This may be explained by the fact that the CCMMD-2R lever arm is not perfectly straight or that the size of the working stroke is restricted by contacts between the motor domain and the α-actinin repeats. However, any major steric clash between the α-actinin repeats and the motor domain is expected to affect the kinetics of product release and thus the steady-state rates. As CCMMD-2R displays high V_{max} (see below), any steric conflicts between the domains are due to be minor in nature.

ATPase activity

The Mg^{2+}-ATPase activity of CCM has not been quantitatively measured due to the very low yields and low purities of native CCM from *Chara* cells, although it was qualitatively shown that addition of actin enhanced its Mg^{2+}-ATPase activity by about 100-fold [4,6]. Therefore, we measured the ATPase activities of recombinant CCM in detail. In the absence of actin, the basal Mg^{2+}-ATPase activities of CCMMD and

Table 1
Steady-state ATPase activities[a]

	Mg^{2+}-ATPase	Mg^{2+}-ATPase + 24 μM actin
CCMMD	0.70 ± 0.02	210 ± 6
CCMMD-2R	1.1 ± 0.1	290 ± 13

[a] Each datum shows Pi liberated/myosin head/s. Values are averages ± SD of at least three independent measurements from two independent protein preparations. Reactions were performed as described under "Materials and methods" at 30 °C.

CCMMD-2R were 0.70 and 1.1 Pi/s/head, respectively (Table 1).

Because the actin-activated Mg^{2+}-ATPase activities of CCMMD and CCMMD-2R were very high, we were using very low concentrations of CCMMD and CCMMD-2R in the ATPase reaction mixture. Concentrations of CCMMD and CCMMD-2R in the reaction mixture were between 0.1 μg/ml (in the presence of 4 mg/ml actin) and 0.5 μg/ml (in the presence of 0.125 mg/ml actin) (see Material and methods). Concentrations of ADP, which are generated by ATP hydrolysis and inhibit ATP binding to motor proteins, were below 0.1 mM during the reaction. Addition of 24 μM actin to CCMMD and CCMMD-2R enhanced their Mg^{2+}-ATPase activity by 300- and 270-fold, respectively. V_{max} and K_{app} values of the actin-activated Mg^{2+}-ATPase of CCMMD and CCMMD-2R were determined by measuring the dependence of the activation on actin concentration and fitting the data to the Michaelis–Menten equation (Fig. 5). V_{max} of CCMMD and CCMMD-2R were 420 and 500 Pi/s/head, respectively. K_{app} of CCMMD and CCMMD-2R were 23 and 19 μM, respectively.

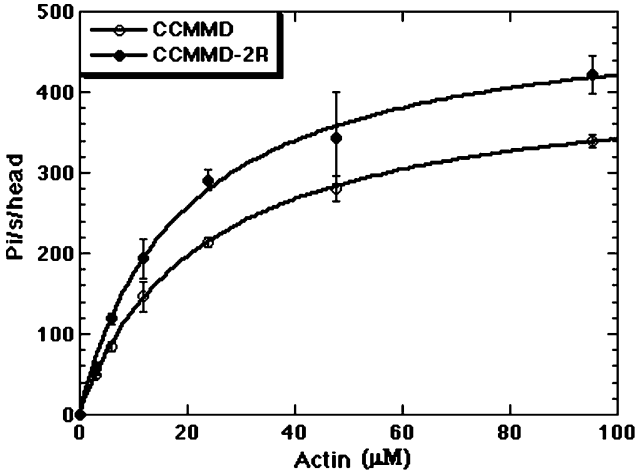

Fig. 5. Mg^{2+}-ATPase activities of CCMMD or CCMMD-2R as a function of actin concentrations. Each value, which was obtained by subtracting the basal Mg^{2+}-ATPase activity of each myosin from the measured value, shows net actin-activated Mg^{2+}-ATPase activity. Values are averages of three to four assays. The reaction was done at 30 °C. Data were fit to the Michaelis–Menten equation. V_{max} of CCMMD and CCMMD-2R were 420 and 500 Pi/s/head, respectively. K_{app} of CCMMD and CCMMD-2R were 23 and 19 μM, respectively.

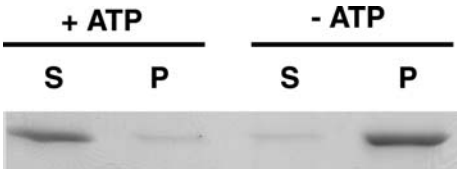

Fig. 6. Cosedimentation of CCMMD with actin. CCMMD (0.5 μM) was cosedimented with 2 μM actin in the absence or presence of ATP. In the presence of ATP (+ATP), >95% of CCMMD were found in the supernatant (S). In the absence of ATP (−ATP), >95% of CCMMD were found in the pellet (P).

We estimated the fraction of dead heads in CCMMD by measuring ATP-dependent acto-MD dissociation. More than 95% of CCMMD cosedimented with actin in the absence of ATP while more than 95% of CCMMD did not cosediment with actin in the presence of ATP (Fig. 6). Therefore, the fraction of dead head was estimated to be less than 10%. This result shows that most of the purified CCMMD is functional and suggests that the measured ATPase activity of the purified CCMMD reflects the actual activity of CCM. The actin-activated Mg^{2+}-ATPase activity of CCMMD and CCMMD-2R was highest among all myosins so far measured. This agrees well with the generality that fast myosins display high ATPase activity [41].

This paper reports the first successful expression and the characterization of the fast myosin from *Chara*. Our results provided conclusive evidence that the cDNA cloned by Kashiyama et al. [7] and Morimatsu et al. [8] encodes the fast myosin in *Chara* cells. Detailed measurements of key kinetic parameters of these motor domain constructs using ensemble kinetics and single molecule analysis will provide more direct estimates for **d** (step size) and $\mathbf{t}_{strong}$ (time spent in the strongly bound state with actin) and will reveal the specific features that allow CCM to move with the highest velocity observed for any myosin studied to date.

Acknowledgments

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