Myosin motors with artificial lever arms

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The myosin head consists of a globular catalytic domain and a light chain binding domain (LCBD). The coupling efficiency between ATP hydrolysis and myosin-induced actin movement is known to decline as the LCBD is truncated or destabilized. However, it was not clear whether the observed alteration in the production of force and movement reflected only the mechanical changes to the length of the LCBD or whether these changes also affect the kinetic properties of the catalytic domain. Here we show that replacement of the LCBD with genetically engineered domains of similar rigidity and dimensions produces functional molecular motors with unchanged kinetic properties. The resulting single-chain, single-headed motors were produced in Dictyostelium discoideum and obtained after purification from a standard peptone-based growth medium at levels of up to 12 mg/L. Their actin motility properties are similar or greater than those of native myosin. Rates of 2.5 and 3.3 μm/s were observed for motor domains fused to one or two of these domains, respectively. Their kinetic and functional similarity to the extensively studied myosin subfragment 1 (S1) and their accessibility to molecular genetic approaches makes these simple constructs ideal models for the investigation of chemo-mechanical coupling in the myosin motor.

Keywords: actin/α-actinin/Dictyostelium discoideum/molecular motor/spectrin repeat

Introduction

All myosins contain a highly conserved globular catalytic domain that contains the actin and nucleotide binding sites and produces force and movement (Cheney et al., 1993; Goodson and Spudich, 1993). This ‘generic’ motor domain is attached via a neck region to a variety of functionally specialized tail domains. The neck region consists of an extended, α-helical region that contains binding sites for one or more calmodulin molecules, or calmodulin-like myosin light chains (Figure 1A and B). The neck region of all conventional myosins binds one essential (ELC) and one regulatory light chain (RLC) and is generally referred to as the light chain binding domain (LCBD). In agreement with previous models (Vibert and Cohen, 1988) and based on the atomic structure of the myosin head (Rayment et al., 1993a) and of the LCBD alone (Xie et al., 1994; Houdusse and Cohen, 1996), it was suggested that the LCBD acts as a rigid lever arm to amplify small conformational changes in the motor domain, thus producing net movement of filamentous actin of ~5–10 nm per ATP hydrolyzed (Rayment et al., 1993b; Schröder et al., 1993). In good agreement with this view, functional studies have shown that the coupling efficiency between ATP hydrolysis and movement declines as the neck region is truncated or destabilized by removal of myosin light chains (Pollenz et al., 1992; Itakura et al., 1993; Lowey et al., 1993; Uyeda and Spudich, 1993; VanBuren et al., 1994; Waller et al., 1995; Woodward et al., 1995). Recently, Uyeda and co-workers produced myosins with neck regions of variable length by adding or truncating light chain binding motifs (Uyeda et al., 1996). As predicted by the swinging neck–lever arm model, this study showed a linear relationship between the number of light chain binding sites and sliding velocity. Support for the lever arm hypothesis was also obtained by cryo-electron microscopy. Three-dimensional maps of myosin-decorated F-actin, obtained in the presence and absence of ADP, show little difference in the region of the globular motor domain; but there is reorientation of the LCBD, compatible with a step of at least 3.5 nm (Jontes et al., 1995; Whittaker et al., 1995). While these studies clearly indicate that the neck region acts as a lever, it remains controversial as to whether changes to the LCBD affect the interaction of the myosin motor with actin, binding of nucleotide, hydrolysis of ATP and communication between the actin and nucleotide binding sites.

In order to get more direct insight into the functional role of the LCBD, we engineered myosin head fragments (MHFs) consisting of the globular catalytic domain of Dictyostelium discoideum myosin fused to a structural motif whose dimensions and rigidity are similar to the LCBD. The best site for fusing an artificial lever arm to the catalytic domain was determined by generating a variety of motor domain constructs and screening their functional behavior using pre-steady-state kinetics. The shortest construct which behaved similarly to an S1-like construct was truncated at Arg761 of the D.discoideum myosin sequence (Geeves et al., 1996). The equivalent residue in adult chicken skeletal muscle myosin is Lys782 (see Figure 1A). Dictyostelium discoideum α-actinin repeats were used as artificial lever arms. Each α-actinin repeat (Figure 1C) consists of ~120 residues and is predicted to form a left-handed coiled-coil consisting of three α-helices, thus creating very compact and rigid structural modules of ~6 nm length (Parry et al., 1992; Yan et al., 1993). Expression vectors for the production of two myosin motors with artificial lever arms were
change in the predicted way, if the length of the substituting domain differs from that of the LCBD?

**Results**

*Expression and purification of recombinant myosin motors*

M761-1R and M761-2R were produced with a C-terminal His tag to facilitate purification by Ni$^{2+}$-chelate affinity chromatography (Janknecht et al., 1991; Manstein et al., 1995). Typically, 2 mg of pure protein were obtained from 1 g of cells expressing either construct. The molecular mass of purified M761-1R and M761-2R was determined by electrospray mass spectrometry (ESMS) as 102 658 ± 7 (102 649.6) and 115 783 ± 14 (115 768.4), respectively. Values given in parentheses refer to the predicted molecular mass of the constructs (Figure 2). The S1-like construct M864 was purified as described previously (Manstein et al., 1989).

*Kinetic analysis of the myosin motors*

The biochemical competence of M761-1R and M761-2R was determined using well established transient kinetics methods. The resulting data (Figure 3 and Table I) were interpreted in terms of the mechanism of rabbit skeletal muscle S1 and compared with similar measurements previously made for M864 (Ritchie et al., 1993). In the absence of actin, we measured both the rate constant of ATP binding and the rate constant of ATP cleavage. The rate constants determined for M761-1R and M761-2R deviated by less than a factor of two from those determined for M864. Similarly, the affinity of ATP and ADP for acto-MHF changed by less than a factor of two, while the rate constants for ATP binding to acto-MHF and the maximum rate constant of ATP-induced dissociation of actin showed changes of <10%. Thus both the actin and nucleotide binding sites and the communication between the two sites show no significant changes between the
S1-like M864 with native light chains and the two fusion constructs.

**Direct functional characterization of the myosin motors**

The similarities in the kinetic behavior of M761-1R and M761-2R to the S1-like fragment are also reflected in the *in vitro* motility data. When bound directly to a nitrocellulose-coated surface, M761-1R and M761-2R support actin movement at average rates of 107 and 169 nm/s, respectively (Figure 4A). This compares with a rate of 124 nm/s determined for M864 and of ~130 nm/s reported for two similar fragments (Manstein et al., 1989; Itakura et al., 1993). While the observed difference in the rates of actin movement over M761-1R and M761-2R correlates well with the prediction of the lever arm hypothesis, the significance of this result is compromised by the fact that full-length *D. discoideum* myosin moves at a 10- to 20-fold higher sliding speed (Uyeda and Spudich, 1993). The slower movement of single-headed fragments is generally explained by differences in the attachment of the motor to the surface. Accordingly, when the orientation of M761-1R and M761-2R was optimized using anti-histidine tag antibody mAb 13/45/3 (Zentgraf et al., 1995) attached to the surface, velocities much closer to those observed with native *D. discoideum* myosin were observed. Values of ~2.5 and ~3.3 μm/s were determined for M761-1R and M761-2R at 25°C, respectively (see Figure 4B). Under similar conditions but at 30°C, values of 1.9–3.1 μm/s were reported for native *D. discoideum* myosin (Ruppel et al., 1994; Uyeda et al., 1994).

**Table I. Summary of rate constants**

<table>
<thead>
<tr>
<th>Rate constant</th>
<th>M864</th>
<th>M761-1R</th>
<th>M761-2R</th>
</tr>
</thead>
<tbody>
<tr>
<td>(k_{i+1}) (M^{-1} s^{-1})</td>
<td>9.4 \times 10^5</td>
<td>5.7 \times 10^5</td>
<td>5.3 \times 10^5</td>
</tr>
<tr>
<td>(k_{i+2}) (s^{-1})</td>
<td>24</td>
<td>29</td>
<td>32</td>
</tr>
<tr>
<td>(k_{i+3}) (s^{-1})</td>
<td>1.4 \times 10^5</td>
<td>1.2 \times 10^5</td>
<td>1.25 \times 10^5</td>
</tr>
<tr>
<td>(K_i (M^{-1}))</td>
<td>330</td>
<td>286</td>
<td>170</td>
</tr>
<tr>
<td>(K_{D} (μM))</td>
<td>450</td>
<td>447</td>
<td>650</td>
</tr>
</tbody>
</table>

The mechanism of nucleotide interaction with myosin and actomyosin derived from studies with muscle myosins is shown above the data. Rate and equilibrium constants in bold type refer to acto-MHF and normal type to the myosin in the absence of actin. The parameters are related to the constants obtained from the data in Figure 3 in the following way. From Figure 3A, the slopes of the fitted lines in the insert are equal to \(k_{i+2}\). \(K_{max}\) = \(k_{i+2}\) + \(K_i\). From Figure 3B, the slopes of the fitted lines are equal to \(K_k\); \(K_{max} = k_{i+2}\). \(K_{D}\) was determined from the ADP inhibition of AT-P-induced dissociation of acto-MHF as described previously (Ritchie et al., 1993).

**Discussion**

In order to elucidate the complex molecular processes underlying the transduction of the chemical free energy
A myosin motor complete with a lever arm consisting of just a single polypeptide chain should be much easier to produce and purify. Examination of the high resolution crystal structure of myosin S1 (Figure 1A) shows the LCBD as an independent domain with few contacts between the heavy chain and the ELC other than the IQ repeat target sequence. This makes it relatively straightforward to design C-terminal extensions of the catalytic domain that are predicted to function similarly to the LCBD. The choice of α-actinin repeats as a replacement for the LCBD was made for three reasons: firstly, the modular structure of the repeats facilitates the generation of motors with variable lever arm length; secondly, modeling of the repeat structure was aided by the extensive body of work on spectrin-like repeats; finally, the repeat sequences could be based on the D. discoideum α-actinin gene, thus avoiding any problems caused by the influence of codon usage on expression levels. D. discoideum exhibits a strong preference for AT-rich codons, and the influence of codon usage on the efficiency of protein production has been documented in at least one study (Dittrich et al., 1994).

Modeling the catalytic domain fused to the rigid coiled-coil α-actinin repeat at the C-terminus in place of the LCBD showed that this construct would resemble the S1 structure but that the lever arm would be a little shorter, 6.5 nm compared with 8.5 nm. With two rigidly joined α-actinin repeats attached at the same residue, the predicted length of the lever arm became 13 nm; thus spanning the natural LCBD length between the two constructs. Both recombinant motor proteins could be produced at levels of ~12 mg/l in D. discoideum and rapidly purified to homogeneity by a selective enrichment step for functional myosin motors, followed by Ni²⁺-chelate affinity chromatography (Manstein and Hunt, 1995). Examination by ESMS rather than just by SDS-PAGE confirmed, with resolution better than a single amino acid, that the desired monomeric constructs with the correct sequence and length were produced and purified (Figure 2). These data, together with the high expression levels, also suggest that both M761-1R and M761-2R fold to form the predicted stable structures.

Stopped-flow fast spectroscopy showed that the fusion of one or two α-actinin repeats to the M761 motor domain created molecular motors with kinetic properties nearly identical to those of the S1-like M864. Their actin and nucleotide binding properties and the communication between actin and nucleotide binding sites were little changed (Table I). Furthermore, while the catalytic domain alone shows very poor movement in in vitro motility assays, the attachment of α-actinin repeats as artificial lever arms produced functional motors that translate actin at velocities nearly proportional to the length of the attached lever (Figure 4). These results reveal that the observed changes in the actin motility assay result solely from alterations to the length of the lever arm rather than being an indirect effect on the kinetic properties of the catalytic domain.

A problem common to all actin-based in vitro motility assays, where the myosin is bound directly to a surface, is the unknown way in which the motor is attached. The point of attachment, its flexibility and the freedom of dynamic orientation may also affect translational velocity.
Further, molecules attached in an unfavorable orientation or not able to move freely may still interact with the actin polymers and reduce their velocity. This may explain the very low translational velocities seen for single-headed myosin constructs (<200 nm/s) compared with those with full-length wild-type *D. discoideum* double-headed myosin (2–3 μm/s) which approach those of fast double-headed skeletal myosins (4–8 μm/s). Thus the velocities which we observed with our constructs attached to nitrocellulose (Figure 4A) may reflect artifacts of the *in vitro* assay surface attachment. This conclusion is supported by our results using the C-terminal His tag to orient the motor construct by binding this tag to antibody molecules adhering to the surface. In this way, the motor molecules were attached at the extreme C-terminal end of the lever arm, raised above the nitrocellulose surface and given a large degree of orientational flexibility. As predicted, velocities then obtained were 20-fold higher and similar to those of full-length wild-type myosin (Figure 4B). Indeed, the velocities obtained (at 25°C) correlated with the estimated length of the lever arm from the atomic model: M761-1R, 6.5 nm and 2.5 μm/s, M761-2R, 13 nm and 3.3 μm/s.

Uyeda and co-workers (1996) used a different approach to varying the length of the lever arm. They either deleted one or both of the light chain binding sites or added a second binding site to the double-headed native *D. discoideum* myosin motor with full coiled-coil tail. In this way, they generated constructs with predicted lengths of 2, 6 and 13 nm from the putative fulcrum point of attachment of the LCBD to the start of the S2 region, and compared these with native wild-type myosin, to which they assigned a lever length of 8.5 nm. They used equilibrium ATPase measurements with or without 24 μM actin as indications of biochemical competence and the *in vitro* motility assay to quantify motor function. Binding the full-length myosin motor proteins, presumably via their long coiled-coil tails, directly to the surface, they found (at 30°C) actin velocities between 0.6 and 4 μm/s, directly proportional to the predicted lever arm length. These are close to the results presented here, and their velocity for wild-type myosin with LCBD length of 8.5 nm, 3 μm/s fits well with our data.

Both from inspection of the chicken S1 structure (Rayment et al., 1993a) and from the functional properties observed for point mutations in the interface between the myosin heavy chain and ELC, that are associated with variants of human cardiac hypertrophy (Poeter et al., 1996), one would expect a more critical role in mechano-chemical coupling for this region than observed with our constructs in the *in vitro* actin gliding assay. The most likely explanation for this discrepancy lies in the fact that this assay measures only one parameter of motor function. A motor has to produce force and movement, and the *in vitro* actin motility assay measures only the latter function against almost no load. The actin *in vitro* motility assay thus gives little indication as to how severely a certain mutation may affect the force production by the motor and about its functional competence in vivo.

We have shown that it is possible to replace the LCBD of *D. discoideum* myosin with rigid -actinin constructs of variable length. The kinetics of such fusion proteins are closely similar to those of wild-type and they can be expressed and purified in large quantities. When orientated by binding to antibody, they have *in vitro* properties similar to those of the wild-type myosin, showing that they are fully functional motors, sustaining actin motility at velocities related to the lever arm length. Their high level of expression, ease of purification and functional competence should make such constructs extremely useful for further structural and functional studies of the myosin motor.

**Materials and methods**

**Construction, expression and purification of recombinant myosin motors**

The expression vectors used for the production of the myosin-α-actinin fusion constructs are based on pDXA-3H (Manstein et al., 1995). The constructs were created by linking codon 761 of the *D. discoideum* myoA gene to Lozanne et al.'s (1996) modified α-actinin gene (Neoge et al., 1987). Constructs fused to one central repeat extended to codon 387 and constructs tagged with two repeats to codon 505 of the α-actinin gene. The peptide Leu–Gly–Ser separates the two sequences at the site of fusion, and both constructs are tagged at their C-terminus with the peptide Ala–Leu–(His)4. All oligonucleotide linkers were optimized for *D. discoideum* codon bias. Modeling of the α-actinin repeat structure was performed using the programs PHD (Rost and Sander, 1993) and Bioscope (Bioscope Inc., Palo Alto, CA). DNA manipulations were done as described in Sambrook et al. (1989) and Eigelhoff et al. (1991). M761-1R and M761-2R were overproduced in AX3-ORF2+ cells and purified by Ni2+–chelate affinity chromatography as described previously (Manstein and Hunt, 1995; Manstein et al., 1995). M864 was purified as described by Ritchie et al. (1993).

Electrospray mass spectrometry was performed on a Fisons VG Platform with on-line trapping as described by Atkielen et al. (1995).

**Stopped-flow experiments**

All transient kinetic measurements were made using a Hi-Tech SF-61 stopped flow spectrofluorimeter. The details of this apparatus and its use for measuring the transient kinetics of rabbit S1 and *D. discoideum* MHFs have been described in our previous studies (Ritchie et al., 1993; Woodward et al., 1995). Actin was prepared from an acetic powder of rabbit skeletal muscle and labeled with pyrene iodoacetamide as described previously (Criddele et al., 1985). Experimental conditions were: 20°C, 0.1 M KCl, 5 mM MgCl2, 20 mM HEPES adjusted to pH 7.0.

**Motility assays**

The *in vitro* assay was performed in a manner similar to that described previously (Amos, 1992) but with some modifications. Briefly, a 1.3NA 40× lens was used in epifluorescence with 546 nm excitation (Hg arc) and 580 nm emission band-pass (Omega filter set XF37). Filaments were imaged with a 4× lens on an intensified CCD TV camera (Darkstar 800) and recorded, via an image processor (Hunamatsu Argus 20) and time-date generator, by an S-VHS VCR (JVC BR5800-E). For slow moving filaments (Figure 4A), the TV format was converted from PAL to NTSC by a CVR22 digital converter (Snell and Wilcox). This permitted automated filament tracking using a VP110 digitizer at 1 frame/s utilizing Expert Vision software (Motion Analysis Inc.) running on a 486DX2-66 PC. For additional manual analysis of fast moving filaments (Figure 4B), tapes were replayed from the VCR to the Argus 20 and individual filaments tracked frame-by-frame using a mouse. Velocity was calculated from the positional change and the time code recorded on the tape. All solutions were kept on ice and the microscope stage was stabilized at 25°C.

Antibody-decorated surfaces were generated as follows: 75 μl of mAb 1345/31 (Dianova GmbH, Hamburg), 200 μg/ml in phosphate-buffered saline, was infused into a 50 μl flow cell with a nitrocellulose-coated bottom surface. The cell was placed in a humidifier at 4°C for 60 min to allow the antibody to bind to the surface. It was flushed with 100 μl of bovine serum albumin (BSA), 10 mg/ml in 100 mM KCl and 50 mM HEPES pH 7.2, and incubated for 15 min at 25°C. This was repeated to ensure blocking of vacant protein binding sites on the surface. It was washed with 100 μl of 1 mg/ml BSA in 100 mM KCl and 30 mM HEPES pH 7.2. Then 50 μl of 1 mg/ml M761-1R or M761-2R in HAB (25 mM KCl, 25 mM HEPES pH 7.5, 4 mM MgCl2, 1 mM EGTA, 5 mM dithiothreitol) was infused from each end of the flow-cell channel.
and allowed to bind to the antibody-coated surface for 45 min in the humidifier at 4°C. After washing with 100 μl of 0.5 mg/ml BSA in HAB, 100 μl of 0.5 mg/ml BSA in HAB. Then 100 μl of AB (HAB with 100 μg/ml of glucose oxidase, 20 μg/ml of catalase, 5 mg/ml of glucose and 0.5 mg/ml of BSA, pH 7.5) was infused and the cell transferred to an inverted microscope (Zeiss Axiosvert 35). After focusing and observation of the actin filaments in rigor, motion was initiated by infusing 100 μl of AB containing 2 mM ATP.

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References


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