Different degrees of lever arm rotation control myosin step size

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Introduction

Myosins constitute a large superfamily of actin-based motors (Mooseker and Cheney, 1995; Mermall et al., 1998). They use the chemical energy of ATP hydrolysis to generate directed movement along actin filaments. Crystallographic data, mostly from conventional myosins, indicate that small conformational changes induced in the myosin head domain by different nucleotide states and binding to F-actin are transmitted to a converter domain that undergoes a large rotational movement (Dominguez et al., 1998). Attached to the converter is the elongated light chain binding domain that acts as a rigid lever arm (Rayment et al., 1993; Whittaker et al., 1995; Uyeda et al., 1996; Suzuki et al., 1998; Houdusse et al., 2000; Shih et al., 2000). The light chain binding domain of different myosins differs in length, as it consists of a variable number of IQ motifs that represent binding sites for calmodulin or calmodulin-related EF-hand protein light chains. The step size of conventional class II myosins and of the processive myosin Va has been demonstrated to depend in a linear fashion on the length of the light chain binding domain (Warshaw et al., 2000; Ruff et al., 2001; Purcell et al., 2002).

However, a comparison of reported step sizes for chicken smooth muscle myosin II, Dictyostelium discoideum myosin II, chicken myosin 1a (brush border myosin I), rat myosin 1b, myosin V, and myosin VI revealed that they are not directly related to the proposed lever arm length (Veigel et al., 1999, 2002; Warshaw et al., 2000; Rock et al., 2001; Ruff et al., 2001; Tanaka et al., 2002). Therefore, to explain these different myosin step sizes, either one or more determinant in addition to lever arm length or a completely different mechanism, as proposed by Yanagida and coworkers (Kitamura et al., 1999; Tanaka et al., 2002), have to be invoked. A comparison of the step sizes of different myosins and an analysis of how they are controlled will lead to the identification of the determinants for these differences. Such experiments should also resolve the ambiguity about the role of the light chain binding domain as lever arm.

Rat myosin 1d (Myo1d),* formerly called myr 4, belongs to a still poorly characterized myosin I subclass (Bähler et al., 1994). Mammalian Myo1d has been implicated to play a role in the endocytic pathway at a step between the early endosome and the recycling endosome (Huber et al., 2000). It consists of a myosin head domain, a light chain binding domain

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*Abbreviation used in this paper: Myo1d, myosin 1d.
with two IQ motifs each binding the Ca$^{2+}$-sensor protein calmodulin, and a relatively short tail domain (Bähler et al., 1994). Here, we report on the analysis of the mechanical properties of various Myo1d constructs by single molecule measurements. We could show that this motor translocates actin filaments with an unexpectedly large step size due to a large deduced angular rotation ($\sim$90°) of its light chain binding domain.

## Results and discussion

### Biochemical characterization of Myo1d

To investigate whether rat Myo1d is indeed a motor, and if so, whether its step size is determined by the same parameters as in conventional class II myosins, we expressed three recombinant rat Myo1d motors with different numbers of light chain binding sites in HeLa cells (Fig. 1). For ease of purification, all our Myo1d constructs were FLAG-tagged at their COOH termini. Affinity purification yielded virtually pure Myo1d head domain and Myo1d head domains encompassing either one or two IQ motifs (Fig. 2 A). Endogenous calmodulin was copurified with the rat Myo1d head domains containing one or two IQ motifs (Fig. 2 B and Table I). The molar ratios of calmodulin to rat Myo1d were determined to be 1.03 ± 0.05 for Myo1d head-1IQ and 1.74 ± 0.15 for Myo1d head-2IQ, respectively. No calmodulin was copurified with the Myo1d head domain that contained no IQ motif (Fig. 2 B). All three constructs exhibited actin-activated ATPase activity. In the absence of actin, the ATPase activities were barely measurable (0.01–0.06 s$^{-1}$). However, in the presence of actin, the ATPase activities reached $V_{\text{max}}$ values of 2–3 s$^{-1}$ with $K_M$ values for actin of 35–40 µM (Table I and Fig. 2 C). Notably, the addition of one or two IQ motifs to the Myo1d head did not significantly change ATPase rates and actin affinities.

### Single molecule measurements of Myo1d

Having established that these three Myo1d constructs exhibit biochemical properties of a motor molecule, we tested them for mechanical activities by single molecule measurements using a combined microneedle laser trap system (Ruff et al., 2001). All three constructs were able to produce directed force along actin filaments as monitored by microneedle force measurements. The migration of purified calmodulin (lane 4). (C) Steady-state ATPase analysis of actin-activated ATPase activity. (A) Cell lysate (l), flow-through of FLAG-column (f), and affinity-purified eluate (e) from each construct were separated on a 7.5% SDS-polyacrylamide gel and stained by Coomassie blue. Molecular weights are indicated on the left, constructs of rat Myo1d are given on top. The asterisk highlights the myosin heavy chains. (B) Coomassie-stained gradient gel of purified rat Myo1d proteins demonstrating the copurification of calmodulin. The asterisk highlights the myosin heavy chains. The arrow marks the position of calmodulin as determined by the migration of purified calmodulin (lane 4). (C) Steady-state ATPase rates for all purified rat Myo1d proteins as a function of actin concentration. Data points for Myo1d-head (circles, dashed line), Myo1d-1IQ (diamonds, straight line), and Myo1d-2IQ (squares, dotted line) were fitted to a hyperbolic equation.

### Table I. Biochemical data and step sizes of recombinant Myo1d

| Construct          | Molecules CaM per heavy chain$^a$ | Basal ATPase rate [s$^{-1}$]$^b$ | Actin-activated ATPase rate $v_{\text{max}}$ [s$^{-1}$] | $K_{\text{M, actin}}$ [µM]$^c$ | Step size [nm] (number of single actinomyosin events/number of actin filaments) |
|--------------------|----------------------------------|----------------------------------|-------------------------------------------------------|-------------------------------|---------------------------------------------------------------------------------|
| Myo1d-head         | 0                                | 0.01                             | 2.6 ± 0.1                                             | 38.1 ± 3.2                    | (1,974/11) (1,883/11) (896/5)                                                   |
| Myo1d-1IQ          | 1.03 ± 0.05                      | 0.01                             | 3.1 ± 0.2                                             | 34.4 ± 10.8                   | (93 ± 0.22)                                                                  |
| Myo1d-2IQ          | 1.74 ± 0.15                      | 0.06                             | 2.7 ± 0.3                                             | 39.6 ± 11.2                   | (13.9 ± 0.15)                                                                 |

$^a$Values were obtained from densitometric analysis as described in Materials and methods.
$^b$Basal ATPase rates are myosin activities in the absence of actin.
$^c$Actin concentration at half the maximum rate in the presence of 2 mM ATP.
Figure 3. Single molecule step sizes of different rat Myo1d constructs. (A) Displacement records showing the interaction of a single actin filament with recombinant rat Myo1d molecules encompassing variable numbers of light chain binding sites. For each construct, representative traces of raw needle displacements (top trace) and the corresponding running variance recordings (bottom trace) are shown. During the attachment of a motor molecule, the fluctuation of the bead–filament–needle complex is significantly reduced. The records contain many actomyosin interactions of variable length; in each panel we highlighted one interaction by an asterisk. Attachments occur over the entire range of the free needle positions. According to Molloy and coworkers (Molloy et al., 1995), the positions of several events show a Gaussian distribution shifted for a distance equivalent to the step size. (B) Distribution of events for the different constructs, summarizing the (oriented) results of several measurements. (C) Step sizes are plotted against the putative lever arm lengths of the various rat Myo1d proteins (circles). The putative lever arm length was calculated by assuming an α-helical conformation of the light chain binding domain. The corresponding solid line represents the best fit of a linear regression analysis. For comparison, previously reported data of D. discoideum myosin II (Dd II, squares; Ruff et al., 2001) and chicken smooth muscle myosin II (sm II, triangles; Warshaw et al., 2000) are also included. The Myo 1d-head construct possesses a COOH-terminal linker of five amino acids that contributes 0.75 nm to the putative lever arm helix. FLAG-tag of Myo 1d and (His)₆-tag of D. discoideum myosin II (Ruff et al., 2001) are not included in this calculation. (D) Analysis for possible substep displacements. Example showing synchronized and averaged records from the interaction of a single actin filament with Myo1d-1IQ (n = 69). We detect no substeps with this analysis, as the average beginning and ending positions differ by <1 nm. In agreement with the histogram analysis, this analysis predicts a step size of just below 10 nm.
(Houdusse et al., 1999). We propose that subtle differences in either of the flexible loops will cause differences in the movement and rotation of the converter and the extended α-helix of the attached light chain binding domain. Indeed, a recently reported crystal structure of a class I myosin, D. discoideum myosin-IE, showed that the overall structure of the motor domain is almost identical to myosin II and that subtle differences occur in loop regions connecting the main structural elements. Due to differences in the relay loop, the lever arm position in the prepower stroke state extended ~30° further up toward the pointed end of the actin filament than in myosin II (Kollmar et al., 2002). The movement of the Dd myosin-IE lever arm from the pre-power stroke position found in the crystal structure to a similar post-power stroke position as observed in crystal structures of class II myosins (Fisher et al., 1995; Houdusse et al., 1999, 2000) would result in an ~90° rotation along the longitudinal axis of the actin filament. These observations strongly support our model of a large angular rotation during the power stroke of Myo1d.

As recently shown, step size measurements for processively moving myosins include, in addition to the working stroke, a diffusive movement to the next preferred myosin binding position (target zone; Rock et al., 2001; Veigel et al., 2002). In the backward moving myosin VI, which contains a unique 53-aa residue insert between the converter and light chain binding domain, this diffusive component appears to be quite large (Rock et al., 2001; Spudich and Rock, 2002). Although details of the processive movements of myosins V and VI still need to be worked out, we believe that these observations are not in conflict with the lever arm role of the light chain binding domain of myosins as suggested by Tanaka et al. (2002).

We conclude that the degree of lever arm rotation varies considerably among different myosins, and that it is an important determinant for myosin step size. Both determinants together, lever arm length and degree of lever arm rotation, are sufficient to explain the reported ATP-induced conformational changes or working strokes (often referred to as step size).

**Materials and methods**

**Plasmid construction**

The rat Myo1d head-FLAG cDNA encoding aa 1–697 of Myo1d followed by linker amino acids GSMS and the FLAG-tag sequence DYKDDDDK was cloned into the expression vector pIREShyg (CLONTECH Laboratories, Inc.). The pIREShyg plasmid was modified previously to encompass Smal, NheI, Bsu36I, and EcoRV restriction sites between the BamHI and BstXI restriction sites. Myo1d head-FLAG cDNA was cloned into the Smal and EcoRV restriction sites in a two-step process. A 3' fragment was amplified by PCR and subcloned into pIREShyg. The remaining 5' fragment was excised from pBSK-Myo1d and ligated. To construct the Myo1d head-1IQ-FLAG plasmid (encompassing aa 1–721 of Myo1d followed by a glycine residue and the FLAG epitope) and the Myo1d head-2IQ-FLAG plasmid (encompassing aa 1–743 followed by a glycine residue and the FLAG epitope), 3' fragments were amplified by PCR and inserted into the BamHI/BstXI sites of the Myo1d head-FLAG construct. PCR fragments were verified by sequencing.

**Cell culture**

HTA-1 HeLa cells (Gossen and Buja, 1992) were cultured at 37°C and 5% CO₂ in DMEM supplemented with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were transfected with calcium phosphate precipitation of plasmid DNA. Single colonies were isolated after selection in 200 µg/ml hygromycin for 2 wk and were subcloned. Cells were analyzed for Myo1d construct expression by immunoblotting with the rat Myo1d antibody SA 522. Selection by hygromycin was maintained continuously.

**Protein purifications**

For purification of recombinant proteins, cells were grown in 10–20 culture dishes (Ø 150 mm) to near confluency. Cells were washed twice with PBS (150 mM NaCl, 3 mM Na₂HPO₄, and 1.5 mM KH₂PO₄) and collected by scraping. They were lysed in a buffer (150 mM NaCl, 20 mM Hepes, pH 7.4, 2 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100, 2 mM ATP, 0.1 mg/ml Pefabloc®), 0.01 mg/ml leupeptin, and 0.02 U/ml aprotinin) for 1 h on ice, and cleared by centrifugation at 40,000 g for 30 min followed by ultracentrifugation at 170,000 g for 45 min. The supernatant was mixed with 1 ml FLAG-antibody agarose (Sigma–Aldrich) and incubated for 2 h at 6°C. Agarose beads were washed twice with buffer WP containing 50 mM KCl, 10 mM Hepes, pH 7.4, 2 mM MgCl₂, 1 mM EGTA, 1 mM 2-mercaptoethanol, and 2 mM Na₃HPO₄. Bound protein was eluted by supplementing buffer WP with 125 µg/ml soluble FLAG peptide (Sigma–Aldrich). The eluted protein was dialyzed for 36 h with two buffer changes against buffer WP. Purified proteins were sometimes concentrated using microcon filters (cut-off 10 kD; Millipore). Densitometric analysis of Coomassie-stained protein bands on SDS gels was performed with a laser densitometer (Ultrascan; LKB). Values represent the mean from at least three different preparations. Purified calmodulin was purchased from Sigma–Aldrich. Actin was purified from rabbit skeletal muscle as described elsewhere (Pardee and Spudich, 1982).

**ATPase assays**

Steady-state ATPase activities at 37°C were determined by quantitation of P, release from γ[³²P]ATP as described previously (Stöffler and Bähler, 1998). Assay mixtures contained 30 mM KCl, 10 mM Hepes, pH 7.4, 2 mM MgCl₂, 2 mM ATP, 2 mM EGTA, 1 mM 2-mercaptoethanol, 2 mM Na₃HPO₄, and various actin concentrations. Purified Myo1d constructs were used in a concentration range between 25 and 200 nM. Kinetic values represent averaged numbers derived from at least three different preparations.

**Step size measurements and data analysis**

The combined microneedle laser trap transducer that was used to measure step sizes has been described in detail previously (Ruff et al., 2001). In brief, the actin filament is pulled taught between a small latex bead (d = 1.0 µm) captured in a laser trap and the tip of a fine glass microneedle. The needle serves as sensor for the transient interactions of the filament with single motor molecules attached to the substrate. All single molecule measurements were performed at 23°C (± 0.5°C) in 25 mM imidazol, pH 7.4, 25 mM KCl, 1 mM EGTA, 4 mM MgCl₂, 1 µM ATP, 10 mM DTT, 100 µg/ml glucose oxidase, 18 µg/ml catalase, and 3 mg/ml glucose. Recombinant rat Myo1d proteins were adsorbed directly to the nitrocellulose-coated surface of the recording chamber using 10-min incubations. Alternatively, they were coupled via an anti-FLAG mAb (M2; Sigma–Aldrich) to the nitrocellulose surface. In these experiments, nonspecific binding was reduced by blocking the surface with BSA. Data analysis was performed with custom-written software as described in detail previously (Ruff et al., 2001). The running variance was calculated with a 10-ms window, and individual events were identified based on the reduction of the variance. The positions of all events relative to the zero position of the free needle were plotted in a histogram and oriented. Measurements obtained with several actin filaments and different protein preparations were pooled to obtain large datasets for each construct. To establish if Myo1dmoves in discrete substeps, like Myo1a, we followed the analysis approach outlined by Veigel et al. (1999), and determined the average position at the beginning and end of the actomyosin interactions. In brief, we determined the beginning and end of individual actomyosin interactions by monitoring the reduction in the variance of a 5-ms running window, and averaged many record segments at the beginning (consisting of 100 ms before and 15 ms into the detected interaction) and separately averaged the end of many individual actomyosin interactions (last 15 ms of the actomyosin event and the 100 ms segment directly after the detachment of myosin). If the step displacement is generated in distinct substeps, positionally synchronized, average beginning and ending event positions will differ. The analysis is principally limited by the bandwidth of the microneedle laser trap detector and the ability to precisely determine the time of the start and end of each interaction in the thermal fluctuations of free actin–microneedle laser trap complex. For the experiments and analysis reported here, we can confidently resolve substeps separated by >3–5 ms.

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