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Allosteric communication in *Dictyostelium* myosin II

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Abstract Myosin's affinities for nucleotides and actin are reciprocal. Actin-binding substantially reduces the affinity of ATP for myosin, but the effect of actin on myosin's ADP affinity is quite variable among myosin isoforms, serving as the principal mechanism for tuning the actomyosin system to specific physiological purposes. To understand the structural basis of this variable relationship between actin and ADP binding, we studied several constructs of the catalytic domain of *Dictyostelium* myosin II, varying their length (from the N-terminal origin) and cysteine content. The constructs varied considerably in their actin-activated ATPase activity and in the effect of actin on ADP affinity. Actin had no significant effect on ADP affinity for a single-cysteine catalytic domain construct, a double-cysteine construct partially restored the actin-dependence of ADP binding, and restoration of all native Cys restored it further, but full restoration of function (similar to that of skeletal muscle myosin II) was obtained only by adding all native Cys and an artificial lever arm extension. Pyrene-actin fluorescence confirmed these effects on ADP binding to actomyosin. We conclude that myosin's Cys content and lever arm both allosterically modulate the reciprocal affinities of myosin for ADP and actin, a key determinant of the biological functions of myosin isoforms.

Keywords Actin · Cysteine · Lever arm · ADP · Fluorescence

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Introduction

Interaction of actin and myosin in the presence of ATP during the actomyosin ATPase cycle generates force and motility in muscle and non-muscle cells. The myosin superfamily consists of multiple isoforms that show differences in biochemical properties that are essential for performing diverse cellular functions (Berg et al. 2001; Foth et al. 2006; O'Connell et al. 2007; Bloemink and Geeves 2011). All myosins have an N-terminal globular head, consisting of the catalytic ("motor") and light-chain domains. The catalytic domain (CD) has binding sites for actin and nucleotide. However, the affinity of myosin for these ligands varies widely across the myosin family.

During its ATPase cycle, myosin goes through multiple steps that change its affinities for actin and nucleotide. While binding of ATP significantly decreases the affinity of every known myosin for actin, resulting in a reciprocal relationship of actin and ATP binding, the affinity of ADP for actin-bound myosin is more variable (Fig. 1). For example, actin decreases the affinity of ADP for fast-moving myosins (e.g., myosin II from skeletal muscle or *Dictyostelium*) by a factor of 100 or more (Greene and Eisenberg 1980; Ritchie et al. 1993), but this factor is less than 10 for slowly moving myosins or strain sensors (smooth muscle myosin, myosin V, or myosin VI) (De La Cruz et al. 1999; Nyitrai and Geeves 2004; De La Cruz et al. 2001; Cremo and Geeves 1998). In the present study,

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to gain insight into the structural basis of this variation, we have studied constructs of *Dictyostelium* (*Dicty*) myosin. Previous kinetic studies using constructs of the CD of *Dicty* myosin II revealed that the effect of actin on the affinity of ADP for myosin depends on the position of the C-terminal truncation. In particular, the sequence 755–761 was found to be essential for maintaining the reciprocal relationship between actin and ADP binding (Kurzawa et al. 1997; Woodward et al. 1995). Another study of *Dicty* myosin showed that this reciprocal relationship could also be decreased by some mutations in the SH1/SH2 helix (Batra et al. 1999). These results indicate that the coupling between actin and nucleotide binding, which is essential for myosin function, can be altered by some, but not all, site-directed mutational changes.

The principal goal of the present study is to further explore the molecular basis of the relationship between actin and ADP binding, using a series of *Dicty* myosin II CD constructs, focusing on the role of cysteines (Fig. 2). Cysteines were selected because (a) in skeletal muscle myosin, Cys707 (SH1) and Cys796 (SH2) are critical for myosin function, and (b) cysteines are widely used in site-directed spectroscopic studies of myosin (Thomas et al. 2009). For several constructs, we started with a Cys-lite (no reactive Cys) version of the 758-aa CD (Korman et al. 2006). This construct expresses poorly unless at least one Cys is added, so the expressed constructs contained Cys at position 619 (S619C, in the actin-binding interface) or 688 (T688C, SH1) or both (678C/T688C, SH2/SH1) (Fig. 2a). A longer construct, adding one IQ domain at the C-terminus of S619C, was used to test the hypothesis of length dependence (Kurzawa et al. 1997; Woodward et al. 1995) (Fig. 2b). Two CD constructs with wildtype Cys content were also tested. Previously characterized M761-2R (Dicty CD fused with two α-actinin repeats) (Kurzawa et al. 1997) was used as a representative of full length wild type (WT) *Dicty* myosin II (Fig. 2). Our results showed that cysteine mutations in the SH1/SH2 helix affect allosteric coupling between ADP and actin-binding.

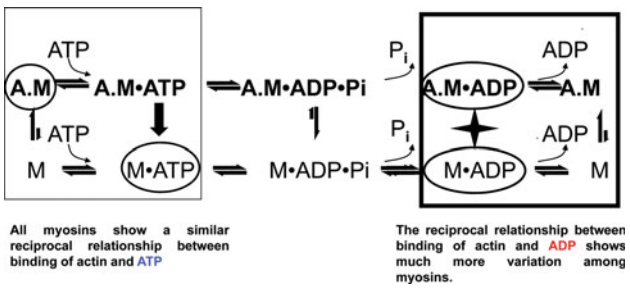

Fig. 1 Actomyosin ATPase cycle. *A* actin, *M* myosin

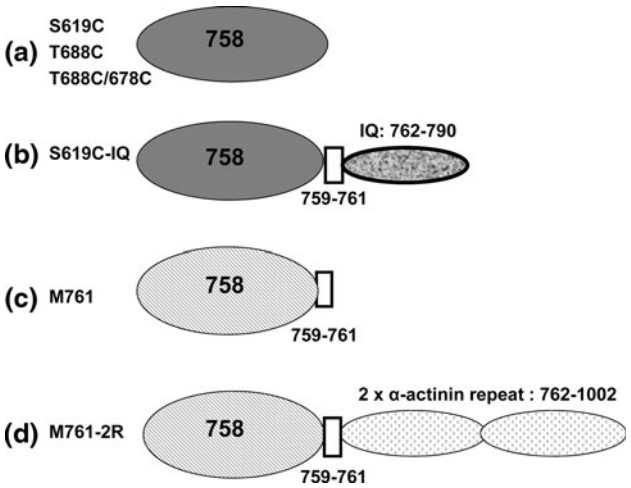

Fig. 2 Schematic representation of *Dicty* myosin constructs used in this study. **a**, **b** based on the 758-aa Cys-lite CD, with Cys substitutions indicated. **b** the S619C construct, extended to include one IQ domain. **c**, **d** based on the 761-aa wild-type CD. **d** M761 fused to two α-actinin repeats

Materials and methods

Purification of actin and labeling

Skeletal muscle actin was prepared as described previously (Prochniewicz et al. 1996) by extracting acetone powder of rabbit skeletal muscle with cold water, polymerizing with 30 mM KCl for 1 h at room temperature, and centrifuging for 1 h min at 200,000×*g*. The pellet was suspended in G buffer (10 mM Tris, 0.5 mM ATP, 0.2 mM $CaCl_2$, pH 7.5). Pyrene-actin was prepared by labeling actin with pyrene iodoacetamide (Invitrogen) as described previously (Criddle et al. 1985), with slight modifications. Actin (48 μM) was polymerized with 0.1 M KCl, 1 mM NaN_3 and 20 mM Tris (pH 7.5), and the dye, freshly dissolved in DMF, was added at a concentration of 96 μM. After 18 h incubation at 23 °C, the labeling was terminated by 10 mM DTT, and actin was ultracentrifuged 30 min at 300,000×*g*. Labeled actin was then resuspended in G-buffer and clarified by 10 min centrifugation at 300,000×*g*. Following polymerization with 2 mM $MgCl_2$ and ultracentrifugation for 30 min at 300,000×*g*, pellets were resuspended in Mg–F-buffer (2 mM $MgCl_2$, 10 mM Tris pH 7.5) containing 0.2 mM ATP. The labeled F-actin was immediately stabilized against denaturation by adding equimolar amount of phalloidin.

Free nucleotide was removed from F-actin by 2 min treatment at 4 °C with 1:3.5 volume of Dowex 1 suspended 1:1 (v/v) in Mg–F buffer, the resin was removed by 2 min centrifugation in a microfuge, and actin was immediately supplemented with phalloidin (0.3 mol of phalloidin to mol of actin) to compensate for the removal of free phalloidin by Dowex 1. Dowex 1 treatment resulted in removal of

more than 99 % of free nucleotide, as determined by measuring the UV absorbance at 260 nm of the supernatant after centrifugation.

Dicty myosin II construction, expression, and purification

A Cys-lite (no reactive Cys) construct of the 758-aa CD was a gift from J. Spudich. This construct expresses poorly unless at least one Cys is added, so the expressed constructs contained Cys at position 619 (S619C) or 688 (T688C) or both (T678C/T688C) (Fig. 2, top) as described previously (Korman et al. 2006). Extension of the S619C construct by adding one IQ domain was made using overlapping PCR of S619C and *Dicty* HMM. M761-2R DNA (two α-actinin repeats fused to the C terminus of a 761-aa wild-type *Dicty* CD) was a gift from D. Manstein. M761 was prepared by truncation of M761-2R. Proteins were expressed and purified as described previously (Manstein and Hunt 1995), except that Talon affinity resin (BD Bioscience) was used rather than Ni^{2+}-NTA, elution was performed in batches of the low-salt buffer with a step gradient of imidazole (30, 50, 100, 200, 300 mM) rather than a continuous gradient. The eluted myosin was then dialyzed overnight at 4 °C against 10 mM Hepes, 0.2 mM EDTA, 1 mM DTT, 50 mM NaCl, and 60 % glycerol, pH 7.5, flash-frozen in liquid N_2 and stored at −80 °C.

ATPase assays

The ATPase assays were performed at 25 °C using the same samples as in the spectroscopic experiments. Ca^{2+}/K^+-ATPase (1.4 μM myosin) was measured in 10 mM $CaCl_2$, 600 mM KCl, 2 mM ATP, 50 mM MOPS (pH 7.5) and the liberated P_i was determined by the malachite green method (Lanzetta et al. 1979). Actin-activated ATPase was measured in Mg–F-buffer containing 2 mM ATP at constant concentration of myosin (0.1 μM) and increasing concentrations (10–50 μM) of nucleotide-free F-actin using NADH-coupled assay, with oxidation of NADH monitored by absorbance at 340 nm (De La Cruz et al. 2000, 2001; De La Cruz and Ostap 2009). The actin dependence of ATPase activity was fitted with a hyperbolic function using Origin 8.0 (Fig. 3).

Binding of MANT-ADP to myosins and actomyosins

Binding of MANT-ADP (Invitrogen) to myosin was detected by measuring sensitized emission of the MANT fluorophore (acceptor) at 440 nm using myosin tryptophans as donors with excitation at 290 nm. Increasing concentrations of (0–20 μM) MANT-ADP were added to 1 μM myosin with or without 2 μM nucleotide free F-actin.

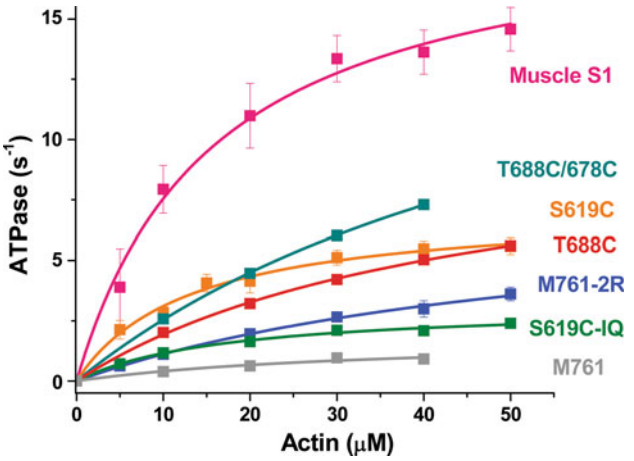

Fig. 3 Actin-activated ATPase activity of myosin constructs. Data were fitted by the function $V = V_{max}$ [actin]/(K_{ATPase} + [actin]), with V_{max} and K_{ATPase} summarized in (Table 1)

Background fluorescence for each concentration of the added MANT-ADP was determined as the sum of emission intensity of free MANT-ADP and emission intensity of myosin ± actin in the absence of MANT-ADP. The fraction of bound was calculated as $y = (F - F_0)/F_{max}$ where F = fluorescence intensity of man-ADP in the presence of myosin construct, F_0 = background fluorescence, and F_{max} = fluorescence intensity corresponding to saturating amount of bound MANT-ADP, obtained by fitting $y = F - F_0$ to the Michaelis–Menten equation. The dissociation constant K_d of MANT-ADP for myosin was determined by fitting the fraction of bound nucleotide y to the quadratic equation

$$y = \left\{ (N_t + M_t + K_d) - \left[(N_t + M_t + K_d)^2 - 4 \times M_t \times N_t \right]^{1/2} \right\} \Big/ (2 \times M_t) \quad (1)$$

where N_t = concentration of added MANT-ADP, M_t = concentration of myosin construct.

To determine directly the amount of myosin-bound MANT-ADP in the absence of actin, 10 μM MANT-ADP was mixed with 2 μM myosin, and the unbound nucleotide was removed by filtration with a Zeba spin column pre-equilibrated with Mg–F-buffer. To determine directly the amount of MANT-ADP bound to myosin in the presence of actin, 10 μM MANT-ADP was added to the actomyosin sample (2 μM myosin + 4 μM nucleotide-free F-actin), the unbound nucleotide was removed by 15 min ultracentrifugation at 300,000×*g*, and the pellet was suspended in Mg–F-buffer. Myosin-bound MANT-ADP was released from proteins by precipitation in 5 % PCA, and its concentration was determined by measuring fluorescence at 440 nm upon excitation at 350 nm.

Interaction of myosins with pyrene-actin

Experiments were performed at 25 °C in Mg–F-buffer. Strongly bound complexes were formed by adding 2.4 μM pyrene-actin with 2.4–10 μM myosin. The fluorescence intensity (excitation 350 nm, emission 407 nm) of 2.4 μM pyrene-actin with different concentrations of myosin was monitored in the absence and presence of 0.1–2 mM ATP.

Each result below is reported as mean ± SEM.

Results

ATPase activities of myosin constructs

All *Dicty* myosin constructs bound strongly to actin in the absence of nucleotide, as measured by cosedimentation, and all were completely dissociated from actin in the presence of ATP, a requirement in the S1dC purification strategy. The high-salt Ca^{2+}/K^{+}-ATPase rates were all within the expected range from 5 to 9 s^{-1}, and basal Mg-ATPase activities were low and all similar ($\sim 0.1 \pm 0.02$ s^{-1}). All constructs were enzymatically active, as determined by actin-activated Mg-ATPase assays (Fig. 3; Table 1). We used an NADH-coupled assay with an ATP-regenerating system that prevents the accumulation of ADP (De La Cruz et al. 2000). The ATPase rates varied substantially among the constructs, both in terms of V_{max} (the maximal activity at saturating actin) and K_{ATPase} (the actin concentration giving $V = V_{max}/2$), resulting in wide range of catalytic efficiencies (V_{max}/K_{ATPase}) (Fig. 3; Table 1). When ATPase activity was measured by P_i release without an ATP-regenerating system (Lanzetta et al. 1979), muscle acto-S1 gave the same results, but the cysteine-substituted *Dicty* constructs showed substantially lower activity than with the NADH-coupled assay (data not shown), presumably because of the accumulation of ADP in the P_i assay.

Table 1 Steady-state actin-activated ATPase activities of myosin constructs

Myosin	V_{max} (s^{-1})	K_{ATPase} (μM)	V_{max}/K_{ATPase} (s^{-1} μM^{-1})
S619C	7.2 ± 0.3	13.0 ± 1.7	0.55 ± 0.05
S619C-IQ	3.2 ± 0.2	17.3 ± 2.5	0.18 ± 0.03
T688C	10.6 ± 0.4	45.3 ± 2.3	0.23 ± 0.02
T688C/678C	19.2 ± 0.5	65.4 ± 2.4	0.29 ± 0.02
M761	1.8 ± 0.6	35.1 ± 21.3	0.05 ± 0.02
M761-2R	7.7 ± 0.7	59.0 ± 8.2	0.13 ± 0.02
Muscle S1	19.5 ± 1.0	15.8 ± 2.3	1.23 ± 0.18

Interaction of myosin constructs with ADP and actin

ADP binding was detected using sensitized fluorescence emission of MANT-ADP while exciting myosin's multiple tryptophans (Fig. 4a) (Woodward et al. 1991). The fluorescence intensity of MANT-ADP bound to myosin was determined by subtracting the fluorescence of free MANT-ADP, actin, and myosin. MANT-ADP was varied to ensure saturation. The fluorescence intensity of saturating MANT-ADP was specific to the myosin construct, decreasing in the order: muscle S1 > M761-2R = M761 > T688C/678C > S619C > S619C-IQ (Fig. 4b). This variation is not due to variable levels of nucleotide binding, since direct determination of bound nucleotide (see methods) showed that muscle S1 and S619C-IQ, which exhibit the largest difference in fluorescence, both bind MANT-ADP stoichiometrically (1.11 ± 0.01 and 0.96 ± 0.38 mol of MANT-ADP/mole myosin, respectively). Thus, the observed

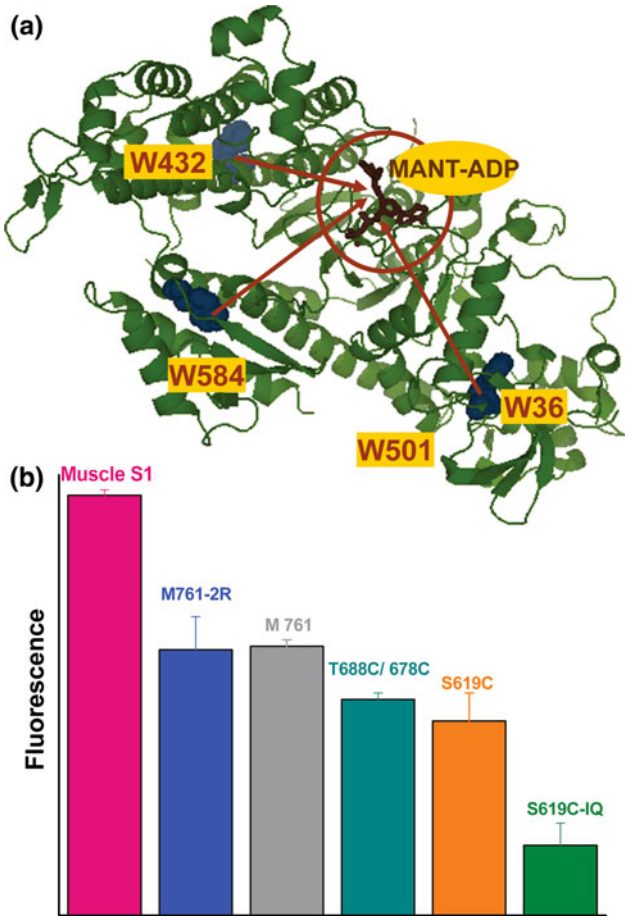

Fig. 4 Sensitized fluorescence emission of MANT-ADP bound to myosin constructs in the absence of actin. **a** Crystal structure (1LVK) of *Dicty* myosin CD, showing location of Trp (W) residues (FRET donors) and MANT-ADP (acceptor). **b** Fluorescence at saturating MANT-ADP (excitation 290 nm, emission 340 nm)

Table 2 Dissociation constants of MANT-ADP from myosin and actomyosin, as determined in Fig. 5

Construct	K_d, myosin (μM)	K_d, actomyosin (μM)
S619C	0.8 ± 0.1	1.3 ± 0.3
S619C-IQ	1.1 ± 0.5	1.1 ± 0.4
T688C	0.3 ± 0.1	0.9 ± 0.3
T688C/678C	2.5 ± 0.6	9.2 ± 4.1
M761	1.0 ± 0.3	30 ± 15
M761-2R	2.1 ± 0.2	≥180
Muscle S1	0.27 ± 0.05	≥160

fluorescence differences reflect construct-dependent efficiencies of sensitized emission, suggesting structural differences in the conformation of the nucleotide binding site.

In the absence of actin, both the saturating amount of bound nucleotide (0.9–1 mol MANT-ADP/mol myosin) and K_d (1–3 μM) showed little variation among the constructs (Table 2, second column). The measured affinities of MANT-ADP for M761-2R and skeletal muscle myosin are consistent with previously reported values determined from kinetics (Batra et al. 1999; Woodward et al. 1991; Bauer et al. 1997). Thus, these constructs act as important controls for the mutant constructs examined.

K_d for binding of MANT-ADP to myosin was substantially increased by actin for both M761-2R and muscle S1 (Table 2; Fig. 5a), consistent with previous reports (Geeves 1989; Woodward et al. 1991). In contrast, actin did not have a detectable effect on K_d for single-cysteine constructs (Table 2; Fig. 5b). This result was confirmed by direct determination of MANT-ADP binding to S619C-IQ. Introduction of one native cysteine to the T688C construct at position 678, to generate the T688C/678C double-cysteine construct, increases K_d for actomyosin ninefold compared to the single-cysteine constructs, whereas the presence of all cysteines in M761 increases K_d 30-fold (Table 2). This result demonstrates the importance of C678, a highly conserved cysteine that is in the same position as the reactive cysteine "SH2" (C697) in skeletal myosin the allosteric communication between the ADP and actin-binding sites.

The effects of the elevated ADP affinities of acto-S619C and acto-S619C-IQ on the structural states of these actomyosin complexes were further investigated using pyrene-actin, which is sensitive to the transition from weak to strong binding (Geeves et al. 1986). In the absence of ATP, binding of S619C quenched pyrene fluorescence to a level similar to that of muscle S1 (Kouyama and Mihashi 1981) (Fig. 6), but quenching by S619C-IQ was substantially less (initial fluorescence values in Fig. 6b). This difference cannot be due to impaired binding of S619C-IQ to actin, since the protein bound completely, as determined by SDS

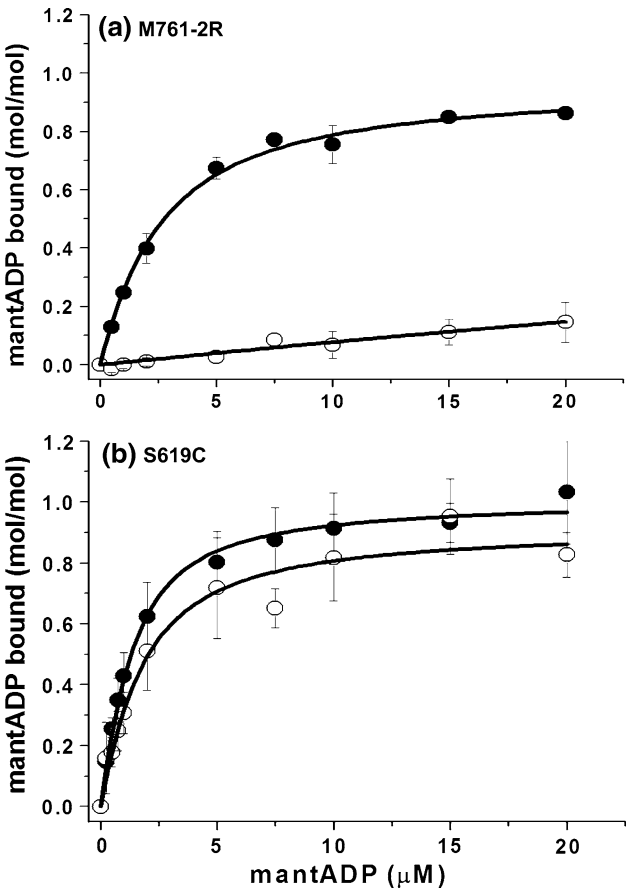

Fig. 5 Binding of MANT-ADP to M761-2R and S619C (1 μM) in the absence (*filled symbols*) and presence of excess (2 μM) actin (*open symbols*). Data were fitted by Eq. 1 to determine dissociation constants (Table 2)

PAGE analysis after cosedimentation (data not shown). We conclude that there is an allosteric effect of the added IQ domain on the structure of the actin-binding interface.

Addition of ATP completely dissociated the actomyosin complex and relieved quenching for all four proteins (Fig. 6). For muscle S1 and M761-R, fluorescence remained at a constant high level for several min (Fig. 6a), characteristic of the weakly bound A.M.ATP complex (Kouyama and Mihashi 1981; Geeves et al. 1986). However, for the two single-Cys constructs, fluorescence immediately began to decrease, and parallel measurements of ATPase activity showed that this decrease occurred before ATP hydrolysis was complete (Fig. 6b). The most likely interpretation is that the high affinity of ADP for the actin-bound single-Cys constructs (Table 2; Fig. 6b) resulted in accumulation of the strongly bound (low fluorescence) A.M.ADP complex. Thus, cysteine substitutions and lever arm truncation both plays an important role in interaction with actin and nucleotide.

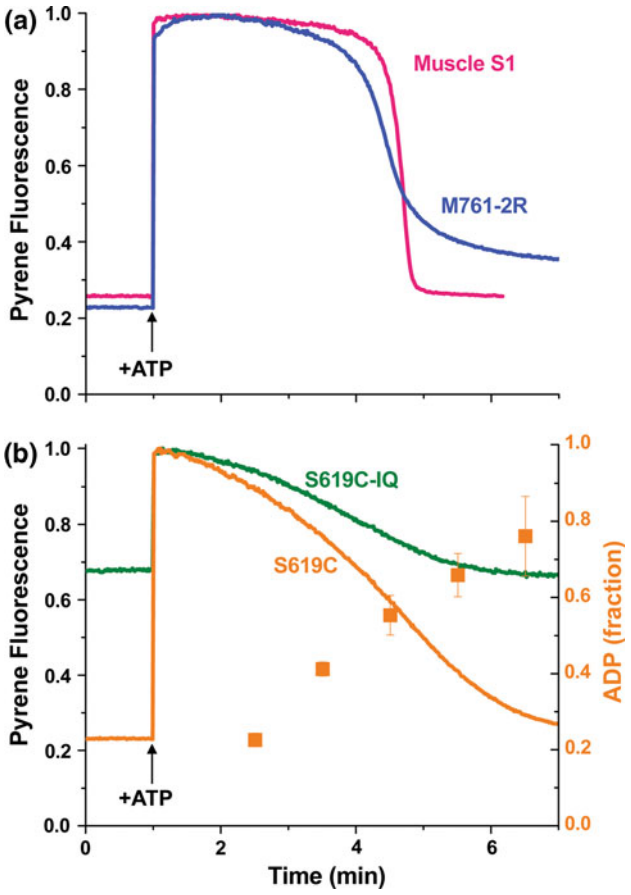

Fig. 6 Interaction of pyrene-actin (2.4 μM) with myosin constructs in the presence of ATP. *Orange squares*: fraction of ADP generated from ATP by the S619C sample. (Color figure online)

Discussion

We report mutational alterations of *Dicty* myosin that have profound effects on the relationship between the binding of actin and nucleotide. While all constructs had significant actin-activated ATPase activities (Fig. 3; Table 1), cysteine substitutions and C-terminal truncations both decreased the reciprocal binding of actin and ADP that is characteristic for wild type *Dicty* myosin (Kurzawa et al. 1997), resulting in accumulation of the strongly bound A.M.ADP complex during the actomyosin ATPase cycle.

Effects of lever arm truncation

The intensity of sensitized fluorescence emission of MANT-ADP bound to myosin depends on the efficiency of fluorescence energy transfer from directly excited tryptophan donors. If all myosins had the same number of tryptophans located at the same distances from the MANT fluorophore, the intensity of sensitized fluorescence would be the same for all of them, but this was not the case (Fig. 4b). The decreased fluorescence in *Dicty* constructs relative to muscle S1 is probably due to the lower content of tryptophan donors in *Dicty* (4) than in muscle S1 (5) and to the difference in conformation between muscle and Dicty active sites (Hiratsuka 1984; Cremo et al. 1990; Franks-Skiba and Cooke 1995; Bauer et al. 1997). However, additional variation among the Dicty constructs (Fig. 4) indicates that the mutations also affect the active-site conformation. The greatest effect comes from the presence of the IQ domain, which suggests coupling between the nucleotide binding cleft and the light-chain-binding domain (lever arm), as previously suggested (Sweeney and Houdusse 2004); (Whittaker et al. 1995). The C-terminal extension affected not only the structural state of the nucleotide-binding pocket but also the affinity of ADP for actomyosin, which significantly decreased after addition of an artificial lever arm to the M761 (Table 2), consistently with previous kinetic studies (Kurzawa et al. 1997).

The state of the lever arm also had substantial effects on the actin activation of myosin ATPase activity (Table 1, Fig. 3). Values of V_{max} and K_{ATPase} were both construct-dependent, resulting in decreasing catalytic efficiency as S619C > S619C-IQ > M761-2R (Table 1). This is consistent with a previous report that the length of the light-chain-binding domain has significant effects on the actomyosin ATPase cycle (Kurzawa et al. 1997), indicating an allosteric effect of the light-chain-binding domain on the interactions with actin and nucleotide. Functional interdependence of the actin-binding and light-chain-binding domains was previously observed for actin-activation of myosin ATPase activity (Wagner et al. 1979) and for in vitro sliding velocity of actin filaments (Lowey et al. 1993) in skeletal muscle myosin. In smooth muscle myosin, actin-activated ATPase is regulated by phosphorylation-induced structural transitions in the N-terminal region of bound light chains (Kast et al. 2010; Himmel et al. 2009), and the activity of molluscan striated muscle myosin as well as cardiac myosin is regulated by Ca^{2+} binding to the regulatory light chain (Himmel et al. 2009).

Effects of Cys mutations in the myosin catalytic domain

We found that some mutations in the *Dicty* myosin CD substantially diminish the reciprocal relationship between the binding of actin and ADP to myosin, which exists in both muscle (Greene and Eisenberg 1980; Siemankowski et al. 1985) and *Dicty* (Kurzawa et al. 1997) (Table 2). The loss of reciprocal binding in S619C has two possible explanations. One is the effect of truncation, since previous studies showed that actin decreases the affinity of ADP for M761 but not for M754 (Kurzawa et al. 1997). However, the anomalously high affinity of ADP for acto-S619C is not

relieved by extending the sequence to residue 790 (S619C-IQ, Table 2). The remaining explanation is a structural perturbation due to the Cys-lite mutations. The role of one of them, C678Y, is supported by the partial rescue of reciprocal binding achieved by adding back this one native Cys (Table 2, T688C/678C). C678 ("SH2") was chosen because it is a highly conserved residue, found in virtually all muscle and nonmuscle myosins, located within the SH1 helix, which is structurally coupled to the state of the nucleotide-binding pocket in both muscle and *Dicty* myosin (Agafonov et al. 2008). In muscle S1, crosslinking two essential thiols Cys 707 ("SH1") and Cys 697 ("SH2") traps bound nucleotide (Wells and Yount 1979) and traps the actomyosin complex in a weakly-bound state (Mello and Thomas 2012). It has also been shown that the G680A mutation in the SH1 helix of *Dicty* myosin eliminates reciprocal binding of actin and ADP (Batra et al. 1999). Among other substituted native cysteines in our study, C599L and C442S are located in upper 50 K domain, which is also structurally coupled to the nucleotide binding cleft. It has been proposed that the movement of this domain, associated with closing of the actin binding cleft, is coupled to opening of switch 1, favoring nucleotide release upon binding of actin (Kurzawa et al. 1997; Houdusse and Cohen 1996; Holmes et al. 2003; Kintses et al. 2007). Substitution C470I in the relay helix could affect coupling of this region to the nucleotide binding cleft (Agafonov et al. 2009). Thus, each of these widely spread cysteine residues, located at structurally important regions of myosin, has the potential to affect intramolecular communications and thus decrease the reciprocal binding of actin and ADP. Further structural studies (e.g., site-directed spectroscopy (Thomas et al. 2009)) will be needed to determine which other native Cys are needed to fully restore reciprocal binding.

Conclusion

In the presence of ATP, the strongly actin-bound state of myosin is predominant for processive myosins such as myosin V and VI and non-muscle myosin II B. *Dicty* myosin II, like skeletal muscle myosin II, is a non-processive motor in large part due to its reciprocal affinity for ADP and actin. We have identified mutations in functionally important regions of *Dicty* myosin II, in both the catalytic and light-chain-binding domains, that substantially reduce this reciprocal affinity, perturbing allosteric communication pathways and providing insight into the structural determinants of myosin isoforms.

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References

Agafonov RV, Nesmelov YE, Titus MA, Thomas DD (2008) Muscle and nonmuscle myosins probed by a spin label at equivalent sites in the force-generating domain. Proc Natl Acad Sci USA 105(36):13397–13402

Agafonov RV, Negrashov IV, Tkachev YV, Blakely SE, Titus MA, Thomas DD, Nesmelov YE (2009) Structural dynamics of the myosin relay helix by time-resolved EPR and FRET. Proc Natl Acad Sci USA 106(51):21625–21630

Batra R, Geeves MA, Manstein DJ (1999) Kinetic analysis of *Dictyostelium discoideum* myosin motor domains with glycine-to-alanine mutations in the reactive thiol region. Biochemistry 38(19):6126–6134

Bauer CB, Kuhlman PA, Bagshaw CR, Rayment I (1997) X-ray crystal structure and solution fluorescence characterization of Mg.2′(3′)-*O*-(*N*-methylanthraniloyl) nucleotides bound to the *Dictyostelium discoideum* myosin motor domain. J Mol Biol 274(3):394–407

Berg JS, Powell BC, Cheney RE (2001) A millennial myosin census. Mol Biol Cell 12(4):780–794

Bloemink MJ, Geeves MA (2011) Shaking the myosin family tree: biochemical kinetics defines four types of myosin motor. Semin Cell Dev Biol 22(9):961–967

Cremo CR, Geeves MA (1998) Interaction of actin and ADP with the head domain of smooth muscle myosin: implications for strain-dependent ADP release in smooth muscle. Biochemistry 37(7):1969–1978

Cremo CR, Neuron JM, Yount RG (1990) Interaction of myosin subfragment 1 with fluorescent ribose-modified nucleotides. A comparison of vanadate trapping and SH1-SH2 cross-linking. Biochemistry 29(13):3309–3319

Criddle AH, Geeves MA, Jeffries T (1985) The use of actin labelled with *N*-(1-pyrenyl)iodoacetamide to study the interaction of actin with myosin subfragments and troponin/tropomyosin. Biochem J 232(2):343–349

De La Cruz EM, Ostap EM (2009) Kinetic and equilibrium analysis of the myosin ATPase. Methods Enzymol 455:157–192

De La Cruz EM, Wells AL, Rosenfeld SS, Ostap EM, Sweeney HL (1999) The kinetic mechanism of myosin V. Proc Natl Acad Sci USA 96(24):13726–13731

De La Cruz EM, Sweeney HL, Ostap EM (2000) ADP inhibition of myosin V ATPase activity. Biophys J 79(3):1524–1529

De La Cruz EM, Ostap EM, Sweeney HL (2001) Kinetic mechanism and regulation of myosin VI. J Biol Chem 276(34):32373–32381

Foth BJ, Goedecke MC, Soldati D (2006) New insights into myosin evolution and classification. Proc Natl Acad Sci USA 103(10):3681–3686

Franks-Skiba K, Cooke R (1995) The conformation of the active site of myosin probed using mant-nucleotides. Biophys J 68(4 Suppl):142S–147S (discussion 147S–149S)

Geeves MA (1989) Dynamic interaction between actin and myosin subfragment 1 in the presence of ADP. Biochemistry 28(14):5864–5871

Geeves MA, Jeffries TE, Millar NC (1986) ATP-induced dissociation of rabbit skeletal actomyosin subfragment 1. Characterization of an isomerization of the ternary acto-S1-ATP complex. Biochemistry 25(26):8454–8458

Greene LE, Eisenberg E (1980) Dissociation of the actin.subfragment 1 complex by adenyl-5′-yl imidodiphosphate, ADP, and PPi. J Biol Chem 255(2):543–548

Himmel DM, Mui S, O'Neall-Hennessey E, Szent-Gyorgyi AG, Cohen C (2009) The on-off switch in regulated myosins: different triggers but related mechanisms. J Mol Biol 394(3): 496–505

Hiratsuka T (1984) Distinct structures of ATP and GTP complexes in the myosin ATPase. J Biochem 96(1):155–162

Holmes KC, Angert I, Kull FJ, Jahn W, Schroder RR (2003) Electron cryo-microscopy shows how strong binding of myosin to actin releases nucleotide. Nature 425(6956):423–427

Houdusse A, Cohen C (1996) Structure of the regulatory domain of scallop myosin at 2 A resolution: implications for regulation. Structure 4(1):21–32

Kast D, Espinoza-Fonseca LM, Yi C, Thomas DD (2010) Phosphorylation-induced structural changes in smooth muscle myosin regulatory light chain. Proc Natl Acad Sci USA 107(18): 8207–8212

Kintses B, Gyimesi M, Pearson DS, Geeves MA, Zeng W, Bagshaw CR, Malnasi-Csizmadia A (2007) Reversible movement of switch 1 loop of myosin determines actin interaction. EMBO J 26(1):265–274

Korman VL, Anderson SE, Prochniewicz E, Titus MA, Thomas DD (2006) Structural dynamics of the actin-myosin interface by site-directed spectroscopy. J Mol Biol 356(5):1107–1117

Kouyama T, Mihashi K (1981) Fluorimetry study of *N*-(1-pyrenyl)iodoacetamide-labelled F-actin. Local structural change of actin protomer both on polymerization and on binding of heavy meromyosin. Eur J Biochem 114(1):33–38

Kurzawa SE, Manstein DJ, Geeves MA (1997) *Dictyostelium discoideum* myosin II: characterization of functional myosin motor fragments. Biochemistry 36(2):317–323

Lanzetta PA, Alvarez LJ, Reinach PS, Candia OA (1979) An improved assay for nanomole amounts of inorganic phosphate. Anal Biochem 100(1):95–97

Lowey S, Waller GS, Trybus KM (1993) Skeletal muscle myosin light chains are essential for physiological speeds of shortening. Nature 365(6445):454–456

Manstein DJ, Hunt DM (1995) Overexpression of myosin motor domains in *Dictyostelium*: screening of transformants and purification of affinity tagged protein. J Muscle Res Cell Motil 16:325–332

Mello R, Thomas DD (2012) Three distinct actin-attached structural states of myosin in muscle fibers. Biophys J 102:1088-1096

Nyitrai M, Geeves MA (2004) Adenosine diphosphate and strain sensitivity in myosin motors. Philos Trans R Soc Lond B Biol Sci 359(1452):1867–1877

O'Connell CB, Tyska MJ, Mooseker MS (2007) Myosin at work: motor adaptations for a variety of cellular functions. Biochim Biophys Acta 1773(5):615–630

Prochniewicz E, Zhang Q, Howard EC, Thomas DD (1996) Microsecond rotational dynamics of actin: spectroscopic detection and theoretical simulation. J Mol Biol 255(3):446–457

Ritchie MD, Geeves MA, Woodward SK, Manstein DJ (1993) Kinetic characterization of a cytoplasmic myosin motor domain expressed in *Dictyostelium discoideum*. Proc Natl Acad Sci USA 90(18):8619–8623

Siemankowski RF, Wiseman MO, White HD (1985) ADP dissociation from actomyosin subfragment 1 is sufficiently slow to limit the unloaded shortening velocity in vertebrate muscle. Proc Natl Acad Sci USA 82(3):658–662

Sweeney HL, Houdusse A (2004) The motor mechanism of myosin V: insights for muscle contraction. Philos Trans R Soc Lond B Biol Sci 359(1452):1829–1841

Thomas DD, Kast D, Korman VL (2009) Site-directed spectroscopic probes of actomyosin structural dynamics. Annu Rev Biophys 38:347–369

Wagner PD, Slater CS, Pope B, Weeds AG (1979) Studies on the actin activation of myosin subfragment-1 isoezymes and the role of myosin light chains. Eur J Biochem 99(2):385–394

Wells JA, Yount RG (1979) Active site trapping of nucleotides by crosslinking two sulfhydryls in myosin subfragment 1. Proc Natl Acad Sci USA 76(10):4966–4970

Whittaker M, Wilson-Kubalek EM, Smith JE, Faust L, Milligan RA, Sweeney HL (1995) A 35-A movement of smooth muscle myosin on ADP release. Nature 378(6558):748–751

Woodward SK, Eccleston JF, Geeves MA (1991) Kinetics of the interaction of 2′(3′)-*O*-(*N*-methylanthraniloyl)-ATP with myosin subfragment 1 and actomyosin subfragment 1: characterization of two acto-S1-ADP complexes. Biochemistry 30(2):422–430

Woodward SK, Geeves MA, Manstein DJ (1995) Kinetic characterization of the catalytic domain of *Dictyostelium discoideum* myosin. Biochemistry 34(49):16056–16064