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Function of the head–tail junction in the activity of myosin II

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ABSTRACT

All class II myosins have the conserved amino acid sequence Pro-Leu-Leu at their head–tail junctions. We systematically altered this sequence in smooth muscle heavy meromyosin (HMM) by site-directed mutagenesis and examined the effects of these mutations on actin–myosin interactions. Deletion of the proline and second leucine did not cause any noticeable change in either actin-activated ATPase activity or actin-sliding velocity. In contrast, deletion of the two leucine residues and substitution of the first leucine with alanine resulted in a 14-fold and 5-fold decrease, respectively, in actin-activated ATPase activity. However, both these mutations did not appreciably affect actin-sliding velocity, which was consistent with a result that there was no considerable change in the ADP release rate from acto-HMM in the deletion mutant. In contrast to double-headed HMM, a single-headed subfragment-1 (S1) with a Leu-Leu deletion mutation exhibited actin activated ATPase activity similar to that by wild type S1. Our results suggest that the first leucine of the conserved Leu-Leu sequence at the head–tail junction profoundly affects the cooperativity between the two heads involved in the actin activated ATPase activity of myosin II.

1. Introduction

Myosin II is composed of a pair of heavy chains, essential light chains and regulatory light chains. The N-terminus of each heavy chain forms a globular structure called S-1 or head, which contains an actin binding site, an ATPase site and two light chains. A long α-helical light chain-binding site together with two light chains forms a rigid bar, which acts as a lever arm to generate movement [1–3]. The remainder of the heavy chain forms a long tail consisting of an α-helical coiled coil structure, which confers the two-headed structure of myosin II.

Recent studies have suggested that one head of myosin II assists the other head by enabling its favorable orientation and optimal interaction with actin, thus enabling its maximal performance. Binding of one head to actin increases the second head's probability of binding to actin [4]. A single-headed myosin II constructed by coexpressing the tail portion of *Dictyostelium* myosin II heavy chain together with the entire heavy chain of myosin II exhibited only half the V_{max} value of the actin-activated ATPase activity per head and half the sliding velocity compared to those with native double-headed myosin II [5]. Tyska et al. proteolytically produced single-headed skeletal and smooth muscle myosins and compared their actin displacement and force generation with those of double-headed ones. Single molecular analysis of these myosins revealed that a double-headed myosin produced twice the unitary displacement and twice the force of a single-headed myosin [6]. Because the difference in the unitary displacement was not due to a successive stroke of the two heads, they suggested that one head merely led the other head to its optimal interaction with actin. A mutation in the nucleotide binding site (E470A) locked myosin in a weak actin-binding state [7,8]. A heterodimeric myosin in which one heavy chain had the E470A mutation and the other heavy chain was that of the wild type exhibited the same step size as wild-type myosin. This suggested that the presence of the first, weakly bound head was necessary to produce maximal displacement of the motion-generating second, strongly bound head [9].

The mechanism of how the first head helps the second head to optimally perform its full function and the nature of head–head interaction are not well understood. Head–head interaction at the N-terminal segment is known to occur when the two heads are bound to actin [10]. The head–tail junction also appears to be critical for both head orientation and the efficient transmission of force produced during the mechanochemical cycle of interaction between myosin heads with actin and ATP.

All myosin II molecules have a highly conserved amino acid sequence (Pro-Leu-Leu) at their head–tail junctions. We systematically altered this region in smooth muscle heavy meromyosin (HMM) by PCR-based mutagenesis and examined the actin activated ATPase activity and the actin-sliding velocity of these mutants. We found that the actin-activated ATPase activity of some of these mutants was considerably lower than that of the

Abbreviations: HMM, heavy meromyosin; S1, subfragment-1; WT, wild type.

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wild type, suggesting the importance of the head–tail junction in actin–myosin interactions.

2. Materials and methods

2.1. HMM constructs

Chicken smooth muscle heavy meromyosin heavy chain in pFastBac (pFastBac his-HMM-myc) was a gift from Dr. Onishi [11]. This construct contains a His-tag at the N-terminus followed by chicken smooth muscle myosin heavy chain from positions 1–1315 and a myc tag. Mutant constructs were generated by PCR based site directed mutagenesis (ExSite, Stratagene). Primers used to generate *AL*, *LA*, *del-LL*, and *del-QV* were CTCTACAGGTCACCCGCC and CCGGCTTCACTTTGGTG, CGCAGGTCACCCGCC and CTAGCGGTT TCACTTTGGTGAACAG, GCGTCAAGAGGAAGAAATGCAGG and GTGA CCTGTGGTTTCACTTTGGTGAACAG, and CGTCAAGAGGAAGAAATG-CAGG and CGTTAGCAGTGGTTTCACTTTGG, respectively.

Subfragment-1 (S-1) constructs were made as follows. pFastBac his-HMM-myc was mutated to create an AgeI site at the down stream region of the nucleotide sequence encoding residue threonine residue 854. These plasmids were cut with SnabI and AgeI and ligated with the SnabI and AgeI fragments of pFastBac CCMMDmyc-his [12]. The resulting construct encoded a His-tag at the N-terminus followed by chicken smooth muscle myosin heavy chain from position 1–854, a linker containing five amino acids (AlaSerGlyGlyGly), a myc tag and a his-tag. Sequences were confirmed using a BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems).

Baculovirus containing mutant HMM and S1 genes were amplified in SF-9 cells and mutants were expressed in High Five cells [12].

2.2. Purification of HMMs

Mutant and wild type HMMs and S1 were purified according to the methods described by Homma et al. [13]. High Five cells were washed and lysed in a buffer containing 1% Nonidet p-40 and HMM or S1. It was coprecipitated with F-actin after ATP hydrolyzes with hexokinase. HMM or S1 was dissociated from F-actin in a solution containing 7 mM MgATP, after which F-actin was removed by centrifugation. HMM in the supernatant was adsorbed to a Ni-NTA agarose resin and recovered using a solution containing 250 mM imidazole. After purification, HMM or S1 was dialyzed against 20 mM KCl, 4 mM $MgCl_2$, 10% glycerol, 1 mM DTT, and 25 mM Hepes–KOH, pH 7.4, at 0 °C. During purification, protease inhibitors (0.1 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml pepstatin, 50 μg/ml TAME, and 40 μg/ml TPCK) were added and all procedures were performed at 0–4 °C.

2.3. Other proteins

Calmodulin was purified according to the method described by Awata et al. [14]. Myosin light chain kinase was purified according to the method described by Ngai et al. [15]. Actin was extracted from the acetone powder of chicken skeletal muscle and purified according to method described by Spudich and Watt [16].

2.4. Phosphorylation of myosin light chain

The regulatory light chain of HMM was phosphorylated as described previously [11]. Phosphorylation was confirmed by SDS–urea gel electrophoresis according to the method described by Perrie and Perry [17] with modifications [18].

2.5. ATPase activity measurement

Steady state ATPase activity was determined by measuring the amount of phosphate released using the method specified by Kodama et al. [19] with modifications [20]. High salt ATPase activity was measured using 0.4 M NH_4Cl, 0.2 M sucrose, 2 mM EDTA, 1 mM DTT, 1 mM ATP, 1 mg/ml bovine serum albumin (BSA), and 25 mM HEPES (pH 7.4) at 30 °C. Actin-activated ATPase activity was measured using 5 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 1 mg/ml BSA, and 25 mM HEPES–KOH (pH 7.4) at 30 °C. Actin concentration varied from 0 to 4 mg/ml.

2.6. In vitro motility assay

An antibody-based version of the *in vitro* gliding filament assay was performed as described previously [12,21]. A flow chamber was prepared using a nitrocellulose coated cover slip and a glass slide as described by Kron et al. [22]. The surface of the flow chamber was coated with anti-human c-myc antibody (0.2 mg/ml in PBS, pH 7.5) for 30 min at room temperature. After antibody adsorption, the chamber surface was blocked with 1 mg/ml BSA and then washed 4 times with HBSS. HMM used to determine motility was mixed with 0.5 mg/ml F-actin in 150 mM KCl and 5 mM ATP and kept on ice for 15 min. The mixture was centrifuged for 10 min at 230,000×*g* and HMM in the supernatant was introduced into the chamber (0.3 mg/ml in HBSS) and kept at 4 °C for 30 min. Before the introduction of F-actin labeled with rhodamine phalloidin, the HMM coated flow chamber was washed with unlabeled F-actin and MgATP to block any residual denatured HMM. The assay buffer used for the *in vitro* motility assay contained 0.8% methyl cellulose, 5 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA, 3 mM ATP, 10 mM DTT, and 10 mM HEPES–KOH (pH 7.4). The *in vitro* motility assay was performed at 25 °C.

2.7. Stopped flow measurement

Stopped-flow measurements were performed using an Applied Photophysics SX18MV stopped-flow spectrophotometer. ATP-induced dissociation of an actin–HMM–ADP complex was determined by monitoring the decrease in light scattering after mixing 0.5 μM HMM, 2.5 μM phalloidin-actin, and 100 μM ADP with 3 mM ATP (final concentration) in the stopped-flow apparatus. Because the equilibrium dissociation constant between ADP and acto-smooth muscle myosin was 5 μM [23], almost all acto-HMM was associated with ADP before mixing. Changes in light scattering were monitored at 90° to the incident light using an excitation wavelength of 310 nm. Assays were done at 25 °C in a buffer containing 5 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, and 10 mM HEPES–KOH (pH 7.4). Data were analyzed using least squares fitting procedure (Kaleidagraph, Abelbeck Software).

3. Results

3.1. Mutant HMMs

The C-terminal half of a myosin II heavy chain has a heptad repeating sequence, which allows this region to dimerize in the form of the α-helical coiled-coil tail [24]. The COILS program provided by Lupas et al. (http://www.ch.embnet.org/software/COILS_form.html, [25]) predicted that the head of chicken smooth muscle myosin II would terminate at Pro-849 and the coiled-coil tail would begin at Leu-850. The Pro-Leu-Leu sequence at the head–tail junction is highly conserved in myosin II (Fig. 1; HMM). As outlined in Table 1, we altered this sequence in smooth

muscle myosin II HMM by site-directed mutagenesis as described in Table 1.

del-P, *del-LL*, and *del-QV* HMMs are deletion mutants in which we deleted Pro, Leu-Leu, and the two residues following Leu-Leu (glutamine and valine), respectively. *Del-P* and *del-LL* HMMs were used to investigate the roles of the conserved Pro and Leu-Leu in the actin–myosin interactions, respectively. *Del-QV* HMM was used as a control for *del-LL* HMM and to investigate whether the deletion of only two amino acids in a nonconserved tail region would affect actin–myosin interaction. *AL* and *LA* HMMs are substitution mutants in which the first and the second leucine residues were substituted with alanine and were used to investigate the roles of the first and the second leucine residues, respectively. These mutations were transfected into insect cells using a baculovirus system and purified from insect cells as described in Section 2.

About 1 mg of protein was routinely obtained from 4 g of wet cells. Purity of these HMMs was determined by SDS polyacrylamide gel electrophoresis; densitometry readings were typically >90%. Purified HMM was treated with myosin light chain kinase (MLCK) before the assay and phosphorylation of the regulatory light chain was confirmed by SDS–urea gel electrophoresis [17] [18].

3.2. ATPase activities

We first investigated the ATPase activity of the mutant HMMs in the absence of actin to see if these mutations exerted any effects on the HMM active site. We determined this activity in the absence of divalent cations but in the presence of 0.4 M NH_4Cl (high salt NH_4 ATPase) because the ATPase activity under this condition is very high and reliable results can be expected [26]. As shown in Table 2, all mutant HMMs had high salt NH_4 ATPase activities similar to that of wild type (WT) HMM in the absence of actin. Thus, these mutations had no effect on the HMM active site.

Next, we determined ATPase activity of the mutant HMMs in the presence of actin. Actin concentration dependence of the MgATPase activity of these mutant HMMs is shown in Fig. 2. Values for *del-P* HMM (V_{max} = 2.3 ± 0.1 Pi/head/s; K_{actin} = 41 ± 6 μM) were similar to those for WT HMM (V_{max} = 3.6 ± 0.2 Pi/head/s; K_{actin} = 61 ± 6 μM), suggesting that the conserved Pro was not very important for actin-activated ATPase activity.

The V_{max} value of *LA* HMM (2.8 ± 0.04 Pi/head/s; K_{actin} = 11 ± 0.5 μM) was close to that of WT HMM, but the K_{actin} value of *LA* HMM (11 μM) was one-sixth of that of WT HMM (62 μM). This showed that *LA* HMM had a much higher affinity for actin than WT HMM.

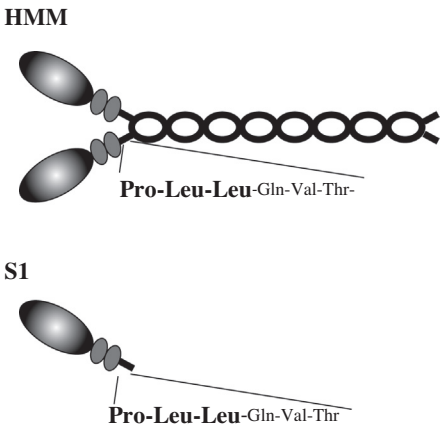

Fig. 1. Schematic diagrams showing the location of the conserved amino acid sequence "Pro-Leu-Leu" in the head–tail junction. This sequence is conserved in almost all myosin II molecules.

Table 1
Amino ascids sequence at the head–tail junction of HMM and S1.

	Mutant name	Sequences
WT		**PLL**QVT
Deletion mutants	*del-P*	**- LL**QVT
	del-LL	**P -** -QVT
	del-QV	**PLL** - - T
Substitution mutants	*AL*	**PAL**QVT
	LA	**PLA**QVT

AL HMM showed a much lower activity than WT HMM and *LA* HMM. *Del-LL* HMM also showed a much lower activity than WT HMM, while values for *del-QV* HMM (V_{max} = 2.8 ± 0.1 Pi/head/s; K_{actin} = 51 ± 5 μM), which was the control for *Del-LL* HMM, were very similar to those for WT HMM. Because the actin concentration dependence of the activity of *AL* and *del-LL* HMMs was almost linear, we could not determine the V_{max} and K_{actin} values for these mutant HMMs. Therefore, we compared these MgATPase activities at an actin concentration of 95 μM; those for *del-LL* and *AL* HMMs were 6% and 19% of that for WT HMM, respectively (Table 2). These results showed that the first leucine of the conserved Leu-Leu was important for the efficient actin-activated ATPase activity of the double-headed HMM.

To investigate whether the conserved Leu-Leu sequence was important not only for the double-headed structure but also for a single-headed structure, we made two S1 constructs: WT S1 and *del-LL* S1 (Fig. 1; S1). MgATPase activity at an actin concentration of 95 μM for *del-LL* S1 (0.23 ± 0.02 Pi/head/s) was similar to that for WT S1 (0.25 ± 0.02 Pi/head/s), which showed that the conserved Leu-Leu sequence was not important for the single-headed structure. These results suggest that the first leucine of the conserved Leu-Leu sequence plays an important role in the cooperative effect of the two heads on actin-activated ATPase activity.

3.3. The time spent in a strongly bound state of del-LL mutant

The rate-limiting step for actin-activated ATPase activity of nonprocessive myosins is the transition step from the weakly bound state with actin to the strongly bound state or the step involving ADP release from the actin–myosin–ADP complex [27–29]. To examine which of these two steps were impaired in *del-LL* HMM, we measured the time spent in a strongly bound state of *del-LL* HMM using a stopped flow technique and this value was compared with that of WT HMM. As described in Section 2, actin-*del-LL* HMM-ADP complex was mixed with ATP and the dissociation of *del-LL* HMM from actin was monitored by light scattering (Fig. 3). The data for the decrease in light scattering was fitted well to an equation with a single exponential term. The dissociation rate of *del-LL* HMM from actin was 49 ± 3 s^{-1}, which was about 80% of that for WT HMM (63 ± 2 s^{-1}), and was far greater than the rate of actin-activated ATPase activity of *del-LL* (Table 2). This result indicated that the ADP release step was not appreciably impaired in *del-LL* HMM and that the low actin-activated ATPase activity of *del-LL* HMM was probably because of a slow transition from the weakly actin-bound state to the strongly actin-bound state.

3.4. In vitro motility assay

Actin sliding velocities for HMMs were determined using an anti-myc antibody-based version of an *in vitro* actin filament gliding assay, in which mutant and WT HMMs were attached to a cover glass with an anti-myc antibody [12,21]. As noted in Section 2, we

Table 2
ATPase activity and actin sliding velocity of WT and mutant HMMs.

	High salt NH_4-ATPase activity (Pi/head/s)	Mg-ATPase activity in the presence of 95 μM actin (Pi/head/s)	Motility (μm/s)
WT	9.7 ± 0.2	2.2 ± 0.1	0.32 ± 0.03
del-P	9.2 ± 0.2	1.6 ± 0.1	0.24 ± 0.03
del-LL	10.1 ± 0.2	0.14 ± 0.01	0.23 ± 0.03
del-QV	9.7 ± 0.1	1.8 ± 0.1	0.32 ± 0.03
AL	10.6 ± 0.3	0.41 ± 0.05	0.22 ± 0.04
LA	11.1 ± 0.2	3.0 ± 0.3	0.36 ± 0.04

Each value was average ± SD of at least three experiments from at least two independent preparations.

added 0.8% methylcellulose to the assay buffer to prevent lateral diffusion of actin filaments. This was because the affinity of *del-LL* for actin in the presence of ATP was very low, as shown in Fig. 2, and smooth actin sliding velocity was not observed without methylcellulose. The motilities of other HMMs were also determined in the presence of methylcellulose. The average sliding velocity of actin filaments with mutant and WT HMMs are shown in Table 2. Unlike the actin-activated ATPase activities, all HMMs exhibited similar motilities. *Del-LL* HMM translocated actin filaments at a velocity of about 70% of that of WT HMM, even though its actin activated ATPase activity was very low. This result was consistent with the results from stopped-flow analysis.

4. Discussion

Both actin activated ATPase activity and the affinity for actin of a double-headed smooth muscle myosin HMM are higher than those of a single-headed myosin S1 [6]. Actin sliding velocity and the unitary step of a double-headed myosin II are also higher than those of a single-headed myosin II molecules produced by genetic engineering [5] or by the proteolytically method [30]. It has been suggested that one head of myosin II helps the other head of the same molecule to bind to actin [9]. The head–tail junction is probably important for the cooperative association of the two heads with actin subunits because the leading head would place the second head in a more favorable position for its interaction with actin.

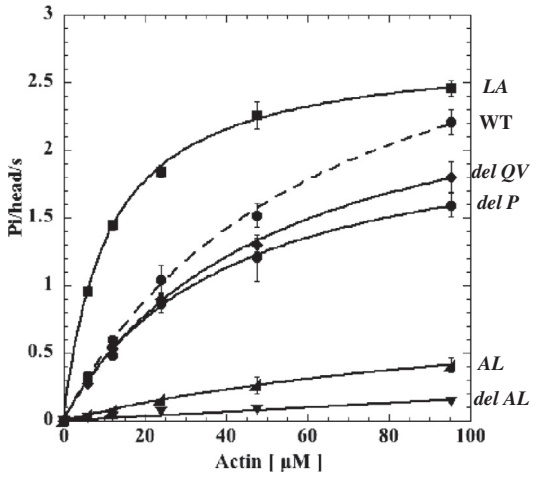

Fig. 2. Actin-activated ATPase activities of WT and mutant HMM smooth muscle myosins. Values are an average of 3 or 4 assays from at least two independent preparations. V_{max} and K_{actin} values of WT, *LA*, *del-QV*, and *del-P* HMMs were determined by fitting data to the Michaelis–Menten equation. V_{max} values for WT, *LA*, *del-QV*, and *del-P* HMMs were 3.6 ± 0.2, 2.8 ± 0.04, 2.8 ± 0.1, and 2.3 ± 0.1 Pi/head/s respectively. K_{actin} values of WT, *LA*, *del-QV*, and *del-P* HMMs were 61 ± 6, 11 ± 0.5, 41 ± 6, and 51 ± 5 μM respectively. Because the actin concentration dependency of *AL* and *del-LL* HMMs activities were nearly linear, we could not determine their V_{max} and K_{actin} values.

The amino acid sequence at the head–tail junction of myosin II is highly conserved; the last amino acid of the head is Pro and the first two amino acids of the tail are Leu-Leu (Fig. 1, HMM). In this study, we investigated the role of these conserved amino acids in the cooperative effects between two myosin heads by site-directed mutagenesis. We showed that *AL* and *del-LL* HMMs exhibited lower actin-activated ATPase activity and had lower affinity for actin than those of WT HMM (Fig. 2 and Table 2). In contrast to the double-headed structure (*del-LL* HMM), the *del-LL* mutant had no effect on the single-headed structure (*del-LL* S1). These results suggest that the first leucine of the conserved Leu-Leu sequence plays an important role in the cooperative association between two heads of myosin II with actin. The difference between the first and the second leucines is in their positions relative to the head; the first leucine is the first amino acid after the head and the second leucine is the second amino acid after the head. Thus, for cooperativity to occur, it is important that leucine is located immediately after the head.

It may be that the side-chain of leucine located immediately after the head (the first leucine) in a double-headed myosin II

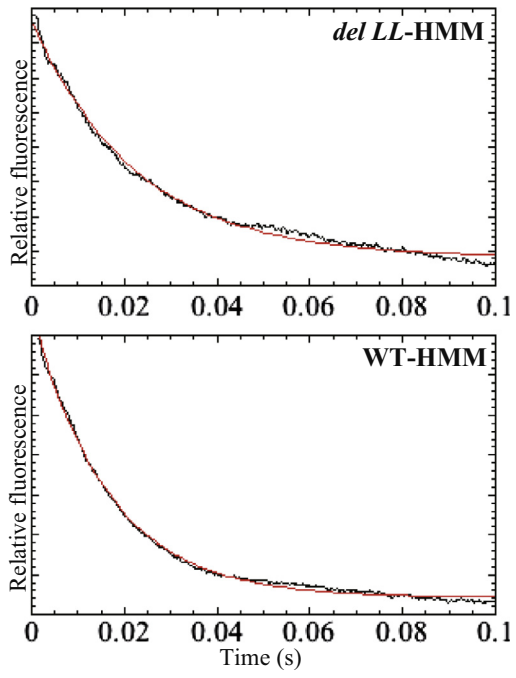

Fig. 3. ATP-induced dissociation of the phalloidin actin-HMM-ADP complex. A stopped-flow apparatus was used to mix 0.5 μM HMM, 2.5 μM phalloidin-actin, and 100 μM ADP with 3 mM ATP (final concentration) and the decrease in light scattering was monitored. The dotted black tracing denotes an average of 4–7 separate recordings and the red smooth line is a fit to an equation with a single exponential term, which gave rate constants of 44 s^{-1} (*del-LL* HMM) and 61 s^{-1} (WT HMM) for the examples shown. The average value of 5 independent assays for two independent preparations of *del-LL* HMM was 49 ± 3 s^{-1} and the average value of 3 independent assays for two independent preparations of WT HMM was 63 ± 2 s^{-1}.

interacts hydrophobically with the side-chain of leucine located immediately after the other head. This hydrophobic interaction between the side-chains of the first pair of leucines may place the two heads in an optimum position for the cooperativity. When this hydrophobic interaction occurs, the hydrophobic interaction between side-chains of the second pair of leucines does not occur, and vice versa, because the angle of the side-chain with respect to the α-helix axis is 100° different between adjacent amino acids.

The hydrophobic interaction between these two pairs may occur alternatively in a myosin II molecule. If this assumption is true, then the hydrophobic interaction between the first pair of leucines in *LA* HMM would be very strong because *LA* HMM does not have the second leucine. In fact, the affinity of *LA* HMM for actin was found to be much higher than that of WT HMM, thus showing high cooperativity between the two heads of *LA* HMM.

Del-P and *LA* HMMs had similar ATPase activities and motilities similar to those of WT HMM (Fig. 2 and Table 2). In addition, as mentioned above, *LA* HMM exhibited a higher affinity for actin than WT HMM. Although no functional activity for the conserved proline and second leucine was found, it cannot be ruled out that these do have some function in full-length filamentous myosin II.

Although the ATPase activity of *del-LL* HMM at a nearly saturated actin concentration was less than 1/10 of that of WT HMM, the motility of *del-LL* HMM was not appreciably impaired (Fig. 2 and Table 2). In accordance with this finding, the time taken by *del-LL* HMM for the strongly bound state with actin was similar to that by WT HMM (Fig. 3). Therefore, the duty ratio (the proportion of the time for the strongly bound state with actin to the ATPase cycle time) of *del-LL* HMM was very small (<1/10 of that of WT HMM) and the number of myosin molecules needed for smooth actin movement for *del-LL* HMM was >10-fold of that for WT HMM. A myosin such as this would be disadvantageous and quite rare in nature, as a large number of these myosin molecules would be required to move an actin filament smoothly.

Acknowledgments

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