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Switch movements and the myosin crossbridge stroke

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Abstract

The myosin II motor from *Dictyostelium discoideum* has been engineered to contain single tryptophan residues at strategic locations to probe movements of switch 1 and switch 2. The tryptophan residue at W501 probes movement of the relay helix and indirectly reports on switch 2 movement. This probe suggests that there is an equilibrium between the switch 2 open- and closed-states when the γ-phosphate position is occupied. Actin does not appear to greatly affect this equilibrium directly, but has indirect influence via switch 1. The latter region has been probed by introducing tryptophan residues at positions 239 and 242. The kinetics of the actomyosin ATPase in solution is discussed with respect to recent crossbridge models based on high-resolution crystal structures.

Introduction

The Lymn-Taylor model (Lymn and Taylor, 1971) brought together low resolution structural data with solution ATPase kinetic results to provide a framework of the actomyosin crossbridge cycle that remains as a central idea in current models of contraction. In this model, the crossbridge stroke was associated with accelerated product release when myosin was bound to actin, while the ATP hydrolysis step occurred while the myosin was detached and acted as a repriming step. Soon after, the hydrolysis step was shown to be freely reversible which meant that little of the free energy of ATP hydrolysis was wasted when the myosin was detached from actin (Bagshaw and Trentham, 1973). Indeed, an equilibrium constant between 1 and 9 for myosin-bound hydrolysis step appears to be a reasonable compromise between ensuring the primed pre-powerstroke conformation is significantly populated without wasting a large proportion of the overall energy of ATP hydrolysis. Taking this argument further is complicated by the need to define the concentrations of the free ligands ATP, ADP and Pi in muscle, as well as the effective actin concentration. The latter is not a single value in a sarcomere but takes on a range of values owing to the structural register between the myosin and actin binding sites. Nevertheless, the low concentration of Pi in muscle (< 1 mM) compared with its apparent binding constant to actomyosin ADP complexes suggested that Pi release is energetically important to drive the overall cycle (White and Taylor, 1976).

The structural details of the Lymn-Taylor model were based on low-resolution electron micrographs from which the crossbridge stroke was assumed to involve a rotation of the whole myosin head whilst it was attached to actin. However, direct evidence for head rotation has been difficult to obtain. Following the high-resolution crystal structure of myosin and the finding that the light chain-binding (neck) domain can take on at least two orientations with respect to the motor domain (Fisher *et al.*, 1995; Smith and Rayment, 1996; Dominguez *et al.*, 1998), it was argued that the bulk of the myosin head did not rotate. Instead, the power stroke was proposed to involve rotation of the neck region: the so-called lever arm hypothesis. Evidence for this idea included the finding of increased sliding velocities and unitary step sizes with increasing lever arm length (Anson *et al.*, 1996; Uyeda *et al.*, 1996), although not all groups reported this relationship (Tanaka *et al.*, 2002). Perhaps the most compelling support is the observation of a change in direction of sliding when the lever arm was bent though 180° by protein engineering but the geometry of the actomyosin interface was left unaltered (Tsiavaliaris *et al.*, 2004).

Molecular details

The nature of this lever arm swing has been defined in increasing detail by comparison of the crystal structures of the myosin with nucleotide bound in the pre- and post-hydrolysis states (Geeves and Holmes, 1999; Holmes *et al.*, 2004). It was evident that with non-hydrolysable ATP analogs, the switch 2 loop was some 4 Å away from the γ-phosphate group so that catalysis

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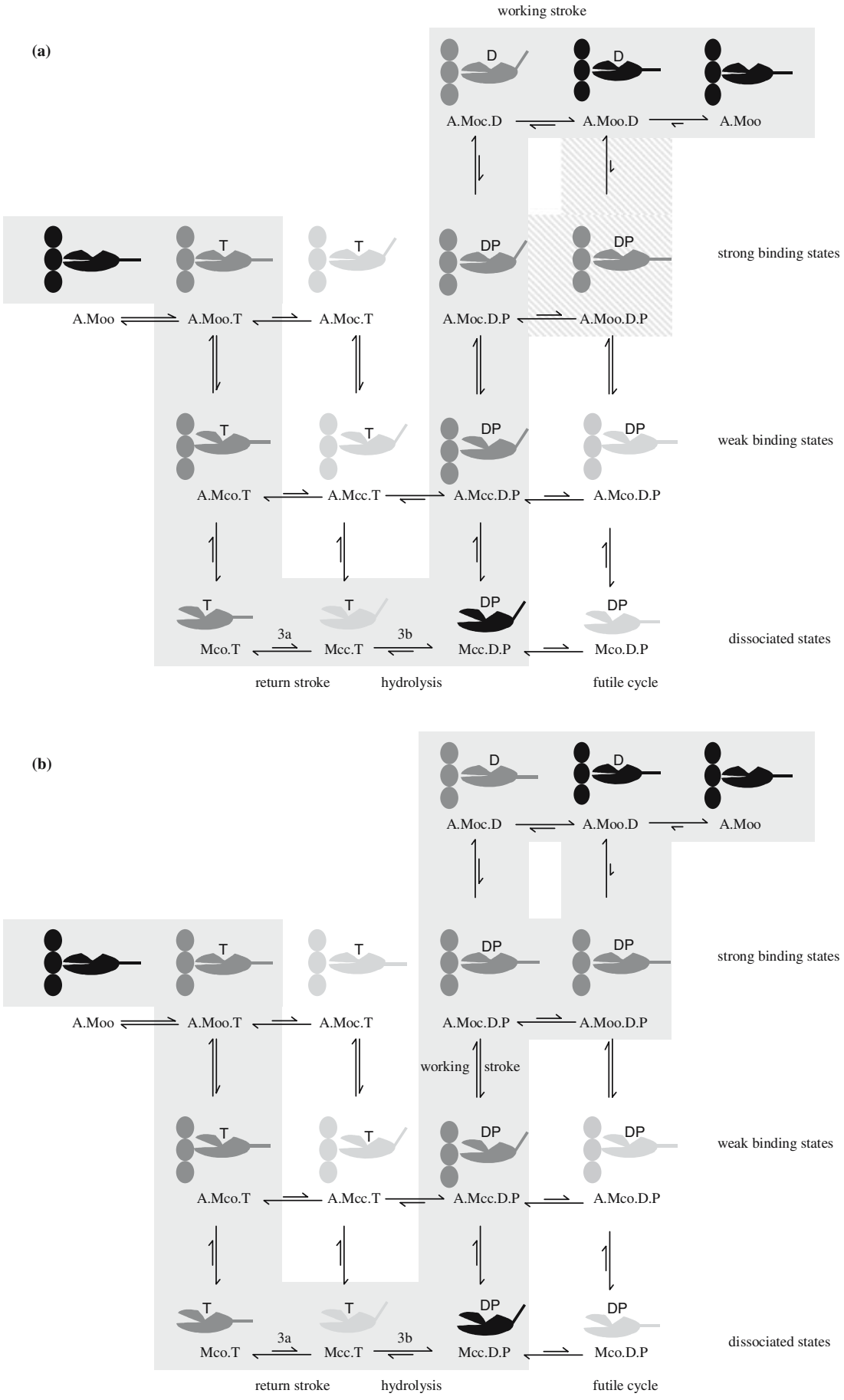
could not proceed. In this conformation the lever arm was extended at an angle to the motor domain, close to that observed when myosin was attached to actin in the rigor state. When the switch 2 loop moves towards the γ-phosphate group, the relay helix to which it attached, moves in unison and this causes the lever arm to rotate through around 60° and trace out an arc on the nanometres scale at its distal end. Recent energy minimalisation calculations suggest that the relay helix movement occurs in two stages – first it see-saws about a pivotal phenylalanine residue in the 20 K subdomain, then the helix kinks by partial unwinding about half way along its length to cause an even greater movement (Fischer *et al.*, 2005). Regardless of the details, switch 2 movement means that hydrolysis can now ensue and the resulting myosin-products complex stabilises the conformation in this so-called pre-power stroke state, ready for interaction with actin. Actin binding somehow reverses this series of events to bring about the powerstroke. However the details of this mechanism are less clear.

Initially it was proposed that actin binding favoured the switch 2 open conformation, so allowing Pi to escape from the active site via the backdoor (Yount *et al.*, 1995; Geeves and Holmes, 1999). Switch 2 opening would be coupled to lever arm movement as for myosin in the absence of actin. However, further structural studies came up with other hypotheses. When actin binds tightly to myosin, the cleft between the upper and lower 50 K myosin sub-domains closes. This idea was proposed in the first attempt to map the crystal structures of the myosin motor domain and actin into the electron density envelope of decorated filaments (Rayment *et al.*, 1993a), and has been supported by more recent analysis (Volkmann *et al.*, 2000; Holmes *et al.*, 2003). In particular, the upper 50 K domain was proposed to move as a relatively rigid body such that closure of the 50 K cleft tends to destroy the interaction of the switch 1 loop with the γ- and β-phosphate of the bound nucleotide (Coureux *et al.*, 2003; Reubold *et al.*, 2003). Thus strong actin binding favours switch 1 opening which itself might be sufficient for Pi to escape from the active site. Once the Pi has dissociated there is no interaction to stabilise switch 2, which then opens and drives lever arm movement as above. This is the basis of the Reubold model (Reubold *et al.*, 2003). Alternatively, once switch 1 opens, switch 2 is destabilised and also opens with accompanying lever movement. Subsequently, Pi is released. More recently, Holmes *et al.* (Holmes *et al.*, 2004) have suggested that the power stroke does not correspond to the reversal of switch 2 opening. Rather they propose that closing the 50 K cleft causes an untwisting of the 7 β-strands in the core of the myosin motor domain such that the pivotal phenylalanine residue moves and relieves its influence on the relay helix (see above). Consequently, the relay helix can unkink and the lever arm can relax back to its extended position before switch 2 opens. These models are shown schematically in Figure 1 and require testing by kinetic methods to ascertain dominant routes in the actomyosin pathway.

Fig. 1. Schematic to link crossbridge states with biochemical states and crystal structures. Myosin is depicted with an open or closed 50 K actin binding cleft and the lever arm in the extended (post-powerstroke) state or bent (pre-powerstroke) state. Black represents stable states, mid-grey represents intermediates that are likely to be on the main pathway but do not dominate (in solution at least), while light grey represent minor species which are more difficult to detect but nevertheless may be on the main pathway. The biochemical states are defined in terms of components (A = actin, M = myosin motor domain, T = ATP, D = ADP, P = Pi) while the subscripts o and c represent the status of switch 1 and switch 2, with switch 1 designated as the first subscript. (a) "Conventional" scheme in which the working stroke corresponds to the switch 2 closed-to-open transition while the motor domain is bound to actin. There remain two possible dominant routes for Pi release. The solid grey pathway corresponds to Pi release before the working stroke (Reubold *et al.*, 2003; Conibear *et al.*, 2004; Zeng *et al.*, 2004) while the striped grey represents Pi release after the working stroke as supported by experiments on muscle fibres (see reviews by Steffen and Sleep, 2004; Takagi *et al.*, 2004). (b) Recent scheme of Holmes *et al.* (2004) in which the lever arm can extend to the post-power stroke state when the 50K cleft is closed, regardless of the status of switch 2. Note in this case the species A.Moc.T and A.Moc.D.P would be expected to have a low W501 tryptophan fluorescence signal equivalent to † rather than * in Equation 1, even though switch 2 is closed. Note that these schematics are oversimplified in that additional conformational changes take place during the release of ADP, and switch 1 and 2 may open further.

Tryptophan fluorescence as a probe

While tryptophan has long been used to probe myosin ATPase states in an empirical way, the fluorescence signal becomes significantly more informative when using constructs containing a single tryptophan residue whose location is defined by the crystal structure (Yengo *et al.*, 1998). The conserved tryptophan located at the end of the relay helix (W501 in *Dictyostelium discoideum* (*Dd*) myosin II) has been particularly informative. Stopped-flow studies indicated that on nucleotide binding there is a 10–20% quench (†) in W501 fluorescence, but for nucleotide analogues containing a γ-phosphate group or equivalent, there is a further rapid enhancement (Malnasi-Csizmadia *et al.*, 2000) that tends to nullify this change (Equation 1). The latter cannot be resolved by rapid mixing methods, but the step is clear in temperature- and pressure jump-experiments (Malnasi-Csizmadia *et al.*, 2001). We interpret these data in terms of rapid but unfavourable closure of switch 2 in the presence of a γ-phosphate group that causes the relay helix to adopt its kinked conformation (step 3a). Only after hydrolysis (step 3b), when the Pi group is free to make a stronger interaction with switch 2, does the equilibrium favour the W501 enhanced state.


(a)
working stroke
D
D
A.Moc.D
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strong binding states
A.Moo
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A.Mco.T
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3a
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return stroke
hydrolysis
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(b)
D
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A.Moo.D
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$$\begin{array}{ccccccccc} M + ATP & \overset{1}{\rightleftharpoons} & M.ATP & \overset{2}{\rightleftharpoons} & M^{\dagger}.ATP & \overset{3a}{\rightleftharpoons} & M^{*}.ATP & \overset{3b}{\rightleftharpoons} & M^{*}.ADP.Pi \\ & & & & & & & & \Updownarrow 4 \\ M + ADP & \overset{7}{\rightleftharpoons} & M.ADP & \overset{6}{\rightleftharpoons} & M^{\dagger}.ADP + Pi & & & \overset{5}{\rightleftharpoons} & M^{\dagger}.ADP.Pi \end{array} \quad (1)$$

Although we interpreted these data in terms of the switch 2 open-to-closed transition (Malnasi-Csizmadia *et al.*, 2000; Malnasi-Csizmadia *et al.*, 2001), W501 itself reports the local environment of a tryptophan located at the end of the relay helix, distal to the switch 2 loop. If the pivotal phenylalanine (F652 in *Dd* myosin II) can move to affect the relay helix conformation (Holmes *et al.*, 2004; Fischer *et al.*, 2005) then W501 fluorescence and switch 2 movement will become uncoupled. The single tryptophan construct containing a F461W may provide a more direct measure of switch 2 position (Wakelin *et al.*, 2002) but further analysis is required. W501 is sensitive to ADP binding, but as yet there is no explanation for this from current *Dd* crystal structures. Some other myosins (e.g. smooth muscle myosin), however, show that ADP can induce a small movement in the lever arm when the motor domain is attached to actin (Sweeney and Houdusse, 2004).

Step 2 in Equation 1 corresponds to the rapid isomerisation(s) that traps the nucleotide at the active site. Tryptophan mutations located near the active site (e.g. D113W, F129W and R131W) are sensitive to step 2, and the F129W construct shows this process comprises at least two sub-steps (Kovacs *et al.*, 2002). ATP and ADP induce similar fluorescence quenches, while pyrophosphate (PPi) induces an enhancement indicating the W129 quench results from adenosine binding. More recently, we have made mutations in the switch 1 region, F239W and F242W (Zeng *et al.*, 2004). These tryptophans are also sensitive to nucleotide binding, but they differ from F129W in their response to nucleotide in that ADP and ATP give differing degrees of fluorescence quench. Furthermore, perturbation methods (temperature- and pressure-jump) indicate that when MgADP, but not ADP, is bound, the W239 probe senses two states in equilibrium which may correspond to different degrees of closure of switch 1. This contrasts with W129 where a single species ($M^{\dagger}$.ADP) dominates at equilibrium. MgATP bound to the F239W construct also appears to give a single species in which switch 1 is presumably fully closed.

We have attempted to study the influence of actin on the switch 1 and switch 2 transitions. Pyrene labels introduced across the 50 K cleft report on the status of the cleft and reveal that myosin cleft movement precedes actin dissociation (Conibear *et al.*, 2003). The pyrene fluorescence amplitudes are consistent with the proposal that apo myosin II has a near open cleft, that closes on binding actin. This implies that the switch 1 is nearly closed in apo myosin (as in the original apo crystal state (Rayment *et al.*, 1993b)). However, the signal from W239 suggests that switch 1 undergoes further closure on binding nucleotide. It is likely that there are several (if not many) substates between the extreme open and closed states of clefts and switches and therefore fluorescence does not necessarily provide a linear measure of the transition between the extremes.

The signal from W129 is useful to monitor the formation of ternary actomyosin-nucleotide complexes (Conibear *et al.*, 2004). For the nucleotides tested (ATP, ATPγS and ADP), actin causes at most a two-fold reduction in the forward isomerisation process (step 2), but ATP induces rapid dissociation so that the ternary A.$M^{\dagger}$.ATP complex does not accumulate. On the other hand, ADP causes only limited and slow dissociation at the protein concentrations used so that a ternary complex dominates for hundreds of milliseconds. ATPγS shows an intermediate response in which the ternary complex exists for tens of milliseconds before significant dissociation occurs, but the latter reaction is near complete. These findings were useful in devising experiments to test the influence of actin on relay helix movements.

The signal from W501 can be monitored in the presence of actin, however the interpretation is complicated. In the case of ATP binding to actomyosin, the actin dissociation rate constant is comparable in magnitude to step 2, so that there is insignificant build up of A.$M^{\dagger}$.ATP to assess the degree to which it equilibrates with A.M*.ATP. This is not the case with ATPγS where both the hydrolysis and actin dissociation reactions are slower. In this case, A.$M^{\dagger}$.ATPγS and A.M*.ATPγS appear to rapidly equilibrate with an equilibrium constant (equivalent to K_{3a}) of about 0.15 compared with 0.26 in the absence of actin (Conibear *et al.*, 2004; Zeng *et al.*, 2004). These values were determined from the level of W501 fluorescence relative to the extreme quench seen with ADP ($K_{3a} < 0.05$) and enhancement seen with ATP ($K_{3a}.K_{3b} > 5$). At 20°C, the actual signal amplitudes observed with ATPγS are small because the equilibrium is poised between the quenched and enhanced states and there is a large static contribution from actin fluorescence. However, we believe the estimate of K_{3a} is valid because at 2°C a clear fluorescence quench was observed when ATPγS was mixed with the W501+ construct in the presence and absence of actin, indicative of $K_{3a} < 0.05$ (Figure 2). Thus the lack of a significant quench or enhancement at 20°C arises from the balance

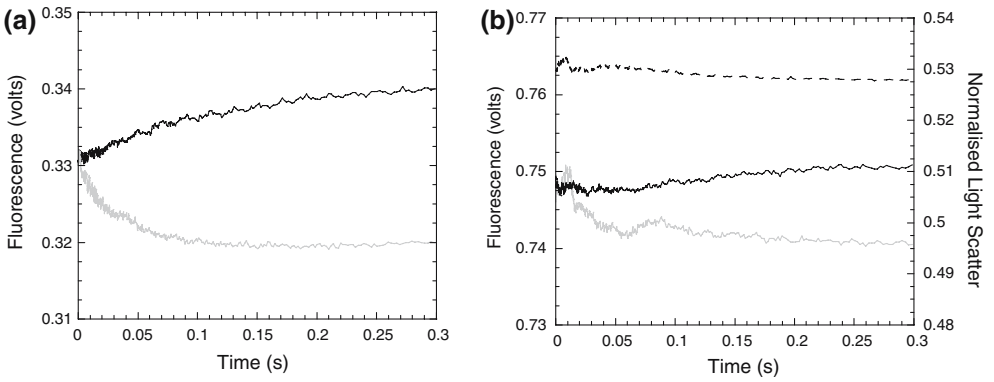

Fig. 2. Stopped-flow, tryptophan fluorescence transients on mixing 100 μM ATPγS with 1 μM W501+ construct in the (a) absence and (b) presence of actin. Reactions were recorded at 20°C (black line) or 2°C (grey line). Because the total fluorescence was higher at lower temperatures, the records were shifted so that their values immediately after mixing were similar. The instrument settings were the same for all experiments and therefore the magnitude of the fluorescence changes are directly comparable. The signals in the presence of actin are higher due to the fluorescence from actin itself, but the magnitudes of the quench at 2°C are similar in the absence and presence of actin (0.01 v), indicative of a near fully open switch 2 state. At 20°C the transient change in the presence of actin is slightly reduced and shows a small lag/quench at the start, consistent with a 2-fold reduction in the open-closed equilibrium constant from 0.26 to 0.15 (Conibear *et al.*, 2004). Under the same conditions in (a), ATP gave an enhancement of 0.38 v endpoint at 20°C and corresponds to about 80% switch 2 closed state (Zeng *et al.*, 2004). The dashed line in (b) represents the light scatter signal, which shows little dissociation on this time scale (the signal decreased to 0.51 after 10 s with a rate constant of 1.5 s^{-1}).

between the two states. Overall, these results indicate that actin has a minor effect on the equilibrium constant of step 3a. Actin did not effect the observed W501 signal in the presence of ADP, but as the equilibrium lies strongly towards the quenched state (nominal switch 2 open) a change in equilibrium constant would not be observed unless it became greater than the detection limit ($K_{3a} > 0.05$).

Implications for switch 1 and switch 2 movements

Previously we argued that the lack of a large effect of actin on the response of W501 fluorescence to nucleotide binding indicates that actin affects a process in the ATPase mechanism other than switch 2 movement (Conibear *et al.*, 2004; Zeng *et al.*, 2004). Switch 1 opening was the most likely candidate. This in turn led to a scheme for actin-activation in which the power stroke followed Pi release. The logic of the argument was as follows. In the actomyosin rigor complex the 50 K cleft is closed, switch 1 and switch 2 are open, and the lever arm is extended (Figure 1). When ATPγS is added at 20°C the binding reaction is fast (as monitored by W129 fluorescence), but the actin dissociated relatively slowly indicating the ternary A.M.ATPγS remains in a switch 1 open, cleft closed conformation for many milliseconds. This conclusion was supported by the response of pyrene probes in the cleft that showed ATPγS gave a slower response than

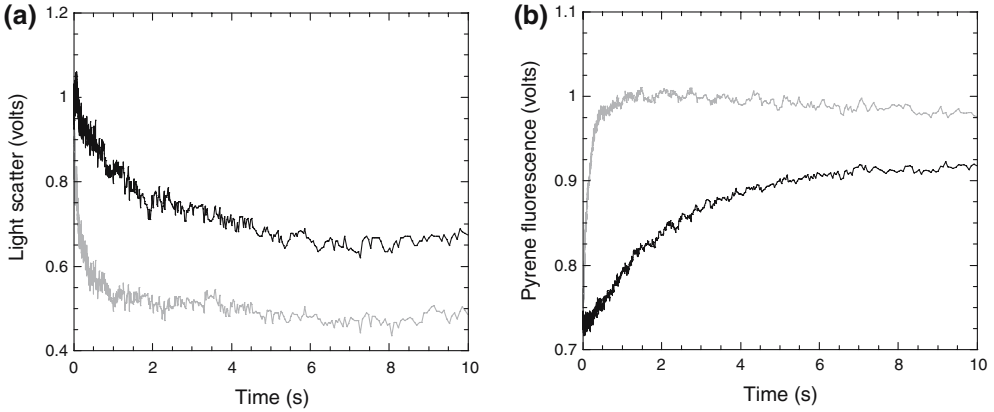

Fig. 3. Stopped-flow records of light scatter and pyrene fluorescence on dissociation of acto-W501+ construct with ATP and ATPγS. (a) 100 μM ATPγS (black line) or 100 μM ATP (grey line) was mixed with 1 μM pyrene-labelled actin plus 1 μM W501+ construct and the light scatter at 400 nm was monitored. (b) The same reaction monitored by pyrene fluorescence with excitation at 365 nm and emission monitored at > 395 nm. Single exponential fits over the dominant phase of the reaction gave rate constants for ATPγS of 0.45 s^{-1} (cf. 0.61 s^{-1} for light scatter) and for ATP of 6.05 s^{-1} (cf. 4.92 s^{-1} light scatter). Buffer conditions: 40 mM NaCl, 2 mM $MgCl_2$, 20 mM HEPES at pH 7.5 and 20°C.

with ATP when the myosin was bound to actin (but the rate constants for these nucleotides were similar in the absence of actin (Conibear *et al*., 2003)). Further evidence is presented in Figure 3 where ATPγS is shown to induce a much slower strong- to weak-binding transition than ATP, as monitored by fluorescence of pyrene-labelled actin. We therefore consider that the A.M.ATPγS complex is initially in a strong binding form (i.e. the cleft remains closed and switch 1 open) while the intermediate level of W501 fluorescence at early times (Figure 2) indicates that the switch 2 equilibrium is similar (within a factor of 2) to that in the absence of actin, where switch 1 is presumed to be closed. Thus the position of switch 2 is dependent on the occupancy of the γ-phosphate site but is not very sensitive to the position of switch 1. However there is evidence against this idea in that the R238-E459 ionic link stabilises switch1 and switch 2 in the closed state (Onishi *et al*., 1998), so that if switch 1 opens, then there is less stabilisation to keep switch 2 closed. Furthermore, Reubold *et al*. (Reubold *et al*., 2003) considered that the R238-E459 link reformed when both switches were considered open, but the assignment of these switch positions depends on what is taken as a reference point within the nucleotide site. If the salt link also forms in the open state then a mixed open-closed state would tend to be even more unfavourable.

Extending our arguments to the products release side, it is well established that the opening of switch 2 in the absence of actin is slow (and probably unfavorable (Wakelin *et al*., 2002)) to account for the long-lived *Dd* M*.ADP.Pi state (i.e. M**.ADP.Pi in skeletal myosin). If actin has little direct influence on switch 2, then switch 1 opening is likely to be the event that is favoured and accelerated by actin binding. Once switch 1 opens and actin is bound tightly (as it must for an effective tension bearing state) in the cleft-closed conformation then either Pi escapes and switch 2 opens or switch 2 opens and Pi escapes. We favoured the former on the grounds that switch 2 opening in the presence of Pi in the γ-phosphate site is unfavourable and is assumed not to be greatly affected by actin, so that the equilibrium would need to be pulled over by Pi release to generate a swing of the lever arm (Conibear *et al*., 2004; Zeng *et al*., 2004). However the order is difficult to define unambiguously. It is possible that opening of switch 1 allows the γ-phosphate group to relocate within the binding site, so allowing switch 2 to relax to its open position, and then Pi escapes.

The recent model of Holmes *et al*. (Holmes *et al*., 2004) proposes that when actin is tightly bound and the cleft is closed, the core β-sheet is twisted so that the lever arm resides in the extended position regardless of the position of switch 2 (Figure 1b). From this it would be expected that the fluorescence of W501 would be quenched on binding nucleoside triphosphate to actomyosin. However, we observe that ATPγS binding to the acto-W501+ construct at 20°C induces a fluorescence signal characteristic of a mixture of the bent and extended lever arm states (Figure 2). Only at 2°C does the bent state become dominant. On switch 1 closing to generate the weak binding state, the β-sheet would untwist, allowing the lever arm to equilibrate to a position similar to that of the value in the absence of actin. If this process is fast ($> 100\ s^{-1}$) and does not limit the dissociation rate ($1.5\ s^{-1}$), then our observed transients does not provide a critical test of the Holmes *et al*. (2004) model. Further studies on switch 1 mutants, such as F239W may help to resolve this ambiguity.

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