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Coupling between phosphate release and force generation in muscle actomyosin

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Energetic, kinetic and oxygen exchange experiments in the mid-1980s and early 1990s suggested that phosphate (P_i) release from actomyosin-adenosine diphosphate P_i ($AM{\cdot}ADP{\cdot}P_i$) in muscle fibres is linked to force generation and that P_i release is reversible. The transition leading to the force-generating state and subsequent P_i release were hypothesized to be separate, but closely linked steps. P_i shortens single force-generating actomyosin interactions in an isometric optical clamp only if the conditions enable them to last 20–40 ms, enough time for P_i to dissociate. Until 2003, the available crystal forms of myosin suggested a rigid coupling between movement of switch II and tilting of the lever arm to generate force, but they did not explain the reciprocal affinity myosin has for actin and nucleotides. Newer crystal forms and other structural data suggest that closing of the actin-binding cleft opens switch I (presumably decreasing nucleotide affinity). These data are all consistent with the order of events suggested before: $myosin{\cdot}ADP{\cdot}P_i$ binds weakly, then strongly to actin, generating force. Then P_i dissociates, possibly further increasing force or sliding.

Keywords: muscle contraction; actomyosin phosphate; force generation; optical trap; single molecule

1. INTRODUCTION

New crystal structures with different forms of myosin, single-molecule mechanical and spectroscopic experiments, and dynamic X-ray diffraction of muscle fibres, are all helping to form an integrated picture of the actomyosin ATPase cycle during contraction. ATP dissociates myosin from actin and is then split to ADP and phosphate. The myosin–products complex attaches to actin, and phosphate release is coupled to generation of the sliding force. In smooth muscle and some unconventional myosins, the rate of cycling seems likely to be controlled by strain dependence of ADP release. But in skeletal muscle, the steps nearer phosphate release at the beginning of the working stroke are likely to control turnover. Here, we consider details of the specific coupling between phosphate release and the transition to the force generating state.

2. HINTS FROM BIOCHEMISTRY

In the Lymn & Taylor (1971) model of muscle contraction, ATP is split by myosin when it is detached from actin. The myosin–products complex ($M{\cdot}ADP{\cdot}P_i$) then binds to actin. A structural change, such as tilting of the myosin head, accompanying release of the hydrolysis products from $AM{\cdot}ADP{\cdot}P_i$, causes the thick and thin filaments to slide. The order of dissociation from $M{\cdot}ADP{\cdot}P_i$ is first orthophosphate (P_i), then ADP (Trentham *et al.* 1972), and the same order applies to product release from $AM{\cdot}ADP{\cdot}P_i$ in solution (White *et al.* 1997) and in muscle fibres (Dantzig & Goldman 1985). In solution experiments with the isolated proteins, the product release steps liberate more than half of the free energy available from the net ATPase reaction, and most of this free energy change corresponds to P_i release (White & Taylor 1976; Siemankowski *et al.* 1985). These observations made P_i release a likely candidate for the main step in the actomyosin ATPase cycle to be coupled to the structural change that produces the sliding mechanical impulse.

The dissociation constant for P_i binding to $AM{\cdot}ADP$ is at least 10^3 M (White & Taylor 1976) and cellular concentrations of P_i are in the millimolar range. Thus, P_i dissociation from $AM{\cdot}ADP{\cdot}P_i$ is nearly irreversible (Sleep & Hutton 1980). But the kinetics of the biochemical steps in a muscle fibre are not expected to be the same as in solution, because the procedures required to isolate and fragment the proteins for transient biochemistry might alter them, the solution conditions are different, particularly the ionic strength, and the mechanical stress and strain present in the muscle filament lattice affect the equilibrium constants of any energy-transducing steps (Goldman & Brenner 1987).

3. EARLY FIBRE EXPERIMENTS

When caged ATP is photolysed to release ATP within a rigour muscle fibre in the absence of Ca^{2+}, the muscle tension and stiffness decline to the low values characteristic of a relaxed fibre. At low photo-released ATP values or when ADP is added, there is a transient tension increase ('bump') as a result of thin filament cooperativity

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(Goldman *et al.* 1982, 1984*a*; Horiuti *et al.* 1994; Thirlwell *et al.* 1994). Addition of P_i accelerates the relaxation of tension and stiffness and suppresses this 'bump' (Hibberd *et al.* 1985*a*). This observation suggested that binding of P_i could detach a force-generating AM·ADP intermediate by reversal of the attachment step.

In the presence of Ca^{2+}, release of ATP activates the fibre, leading to high tension and a stiffness value intermediate between those of relaxation and rigour (Goldman *et al.* 1982; 1984*b*). Addition of 5–10 mM P_i in this situation reduces the stiffness and final force development but accelerates the rate constant of approach to the steady force (Hibberd *et al.* 1985*a*). These observations are also compatible with reversal of the transition leading to the force generating state, because the observed rate constant is the sum of the forward and backward elementary rate constants. If the transition leading to the force generating state is reversed by binding of P_i to AM·ADP, then increasing the P_i concentration would increase the rate of that reverse reaction.

This ready reversibility was a novel idea at the time, because of the known low affinity of P_i to actomyosin in solution. There was earlier evidence for P_i binding and incorporation into ATP in an active muscle fibre (Ulbrich & Rüegg 1976). Experiments using stable isotopes of oxygen and mass spectrometric analysis showed that an active muscle fibre would catalyse the exchange of the oxygen atoms of P_i in the bathing medium with those of the solvent (Webb *et al.* 1986). The pathway for this exchange reaction is binding of P_i to an ADP-containing myosin state (M·ADP or AM·ADP), reformation of ATP, hydrolysis with incorporation of solvent oxygen, and release of the exchanged P_i. Note that this so-called 'medium oxygen exchange' is different from 'intermediate oxygen exchange' between ATP and the solvent (Hibberd *et al.* 1985*b*), which does not require reversal of P_i release. Medium P_i exchange occurred in activated, but not relaxed muscle fibres, implying that the state capable of binding P_i is AM·ADP (Webb *et al.* 1986; Bowater *et al.* 1989). Exchange of this type in acto-subfragment 1 was also lower than in fibres (Bowater *et al.* 1990).

The rate of P_i binding, as evidenced by medium exchange, in activated muscle fibres was, however, only *ca.* 500 M^{-1} s^{-1}, leading to a kinetically estimated dissociation constant for P_i of *ca.* 100 mM, much higher than the range of P_i concentrations that cause suppression of steady force (5–20 mM). These observations imply that P_i can bind to an AM·ADP state in an active muscle fibre, but that only a small proportion of the myosin heads (less than 30%) populate that state at any instant because the rest of the crossbridges are detached or not bearing force.

4. DETAILS OF THE LINK BETWEEN PHOSPHATE RELEASE AND FORCE GENERATION

The phosphate release and the structural transition that leads to the force-generating state are not necessarily simultaneous. In the monograph, '*Muscular Contraction*', A. F. Huxley (1974) hypothesized that mechanical and biochemical steps would alternate in the crossbridge cycle, like events in a clock escapement, imposing an efficient, orderly sequence of transitions. Is phosphate released before, simultaneously, or after the generation of force in the actomyosin ATPase cycle?

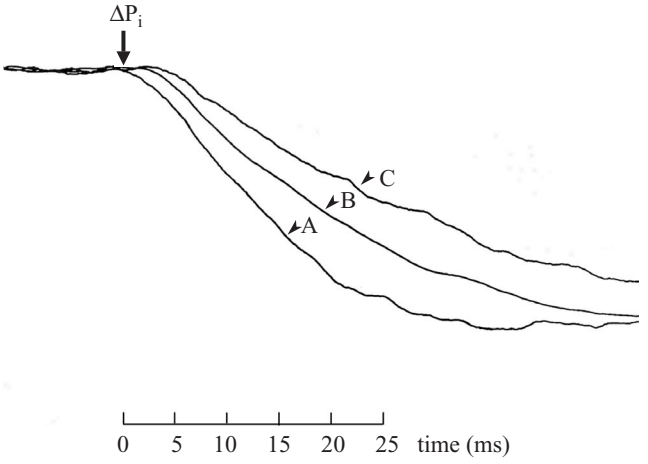
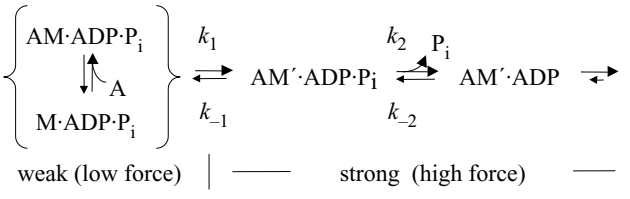


Figure 1. Partial reduction of force generation following rapid release of P_i from caged P_i . Tension recordings from a caged P_i photolysis experiment in a single glycerol extracted rabbit psoas muscle fibre at 10 °C have been scaled to the final amplitude to emphasize the kinetics. The tension transients show two phases: an initial lag phase following the laser pulse (arrow) and a second, nearly exponential phase (arrowheads). The durations of each phase depend only on the final phosphate concentration, here A, 6.8 mM; B, 1.8 mM and C, 0.64 mM. The time scale indicates milliseconds.

Photolysis of caged P_i within an actively contracting muscle fibre causes a decline of force as expected from the reversibility of P_i release (Millar & Homsher 1990; Dantzig *et al.* 1992; Walker *et al.* 1992). The kinetics of the transients initiated by photolysis of caged P_i exhibit several characteristics that bear on the order of the transitions in question. Figure 1 shows force traces similar to those published by Dantzig *et al.* (1992) on glycerol-extracted fibres from rabbit psoas muscle. Release of P_i is very rapid (within 40 μs), but following the laser pulse, there is a lag of several milliseconds before force declines. Then a nearly exponential decline ensues, and subsequent slower transients precede the steady state. At 20 °C, the rate constant for the main, nearly exponential decline is *ca.* 20 s^{-1} at low P_i concentration, saturating at *ca.* 200 s^{-1} at high P_i concentration (Millar & Homsher 1990; Dantzig *et al.* 1992; Walker *et al.* 1992).

The increase in the rate constant and its saturation at high P_i concentrations strongly suggest that the process monitored by this transient occupies more than one reaction step. A simple equation which explains the data is given in Scheme 1.



Scheme 1.

AM·ADP·P_i is considered to be in rapid equilibrium with M·ADP·P_i as indicated by lumping them together within the brackets. In the forward direction the isomerization of

AM·ADP·P_i to AM′·ADP·P_i leads to development of force, and P_i dissociates from AM′·ADP·P_i to form AM′·ADP, stabilizing force generation. During a mechanical transient initiated by photolysis of caged P_i, phosphate binds to AM′·ADP, forming AM′·ADP·P_i without loss of tension (explaining the lag phase of the P_i transient; figure 1) and then the isomerization reverses, building up the population of low-force AM·ADP·P_i and M·ADP·P_i. The rapid exchange between these two states explains the decrease of stiffness of the fibre in the presence of P_i, which is less than the decrease of tension when P_i is added (Kawai & Halvorson 1991; Dantzig *et al.* 1992). Assuming that k_2 is much faster than k_1 and k_{-1} in Scheme 1, P_i binding and dissociation from AM′·ADP·P_i are in rapid equilibrium as is usual for a ligand binding step, the observed rate constant for the tension decline would be given by $k_{obs} = k_{+1} + k_{-1}[P_i]/(K_P + [P_i])$, where $K_p = k_2/k_{-2}$. This expression gives $k_{obs} = k_{+1}$ at low P_i concentration and a hyperbolic increase to $k_{obs} = k_{+1} + k_{-1}$ at saturating P_i concentration, as observed. K_P was estimated to be 3.7 mM at 20 °C and 12.3 mM at 10 °C, in the range of P_i concentrations that reduce steady tension (Dantzig *et al.* 1992).

As long as the elementary P_i binding step itself is a rapid equilibrium, other two-step reaction schemes do not explain these caged P_i results (Dantzig *et al.* 1992). For instance, if AM′·ADP·P_i does not bear force, then the lag phase would not be observed and the exponential rate of the main tension decline would not saturate. If the two reaction steps in Scheme 1 are reversed, with P_i dissociation before force generation in the forward direction, then increasing P_i concentration would decrease the observed rate constant, contrary to what is observed.

Two other types of experiment reported around the same time also concluded that Scheme 1 applies. Kawai and colleagues (Kawai & Halvorson 1991; Kawai & Zhao 1993) measured the frequency-dependent complex modulus of elasticity of rabbit muscle fibres by sine-wave analysis. The rate constant for the intermediate-speed kinetic component of the spectra depended on P_i concentration in the same way as the exponential phase of the caged P_i transients. The tension increase after rapid release of high pressure imposed on a contracting muscle fibre (pressure jump) also has kinetics and dependence on P_i concentration very similar to those of the caged P_i transients (Fortune *et al.* 1991, 1994). Both of these groups favoured Scheme 1, again assuming that the P_i release step is fast.

5. DOUBTS ABOUT SCHEME 1

Development of a sensitive chromophoric probe of P_i release allowed the rate of release from actomyosin to be measured directly. White *et al.* (1997) found that P_i release from actomyosin in solution is only 75 s^{-1}, not sufficiently faster than the ATP hydrolysis step to support the assumption of a rapid equilibrium described above. More complex schemes could, of course, be invoked, and integration of pressure- and temperature-jump and fibre transient data led Ranatunga (1999) to postulate yet another state in this part of the actomyosin cycle. If the 75 s^{-1} value for P_i dissociation measured with isolated acto-S1 applies to experiments in which muscle fibres are activated by photolysis of caged ATP (Goldman *et al.* 1984*b*; Sleep *et al.* 2004), the rate of force development is too fast for it to follow P_i release (Sleep *et al.* 2004). However, the moderate rate of P_i release does call into question the fibre kinetic analysis listed in § 4.

Alternatives to Scheme 1 have also been considered on the basis of crystal structures of myosin. The atomic structure of the myosin head was first solved by X-ray crystallography by Rayment and colleagues using chicken skeletal muscle subfragment 1 in the absence of nucleotide, presumably the state at or near the end of the power stroke (Rayment *et al.* 1993*b*; figure 2*a*). Most of this structure fits well into cryo-electron micrographic maps of actin decorated with myosin heads, providing evidence of the position of the near rigour head relative to the actin filament (Rayment *et al.* 1993*a*; Schröder *et al.* 1993). The head is fairly straight and the light chain domain points towards the barbed end of actin, as expected after the myosin molecule has carried its load (the thick filament in muscle) in that direction (downwards in figure 2*a*). A cleft between the upper and lower 50 kDa domains would need to close to avoid clashing between residues in actin and myosin (Rayment *et al.* 1993*a*). The loop, termed switch II, which contains the invariant glycine that forms a crucial hydrogen bond with the γ-phosphate of ATP, is well away from the position necessary to support ATP hydrolysis. This position of switch II is termed 'open'. A tunnel in the protein, termed the 'back door' provides a potential route for P_i to exit before ADP (Rayment *et al.* 1996).

In the presence of ADP and P_i analogues, vanadate and aluminum fluoride, the myosin head adopts a different configuration, more bent and with switch II in the catalytic position, closer to the P_i analogue (*Dictyostelium* myosin II (Fisher *et al.* 1995; Smith & Rayment 1996) and smooth muscle myosin (Dominguez *et al.* 1998)). This position for switch II is termed 'closed'. The structure in figure 2*b* represents myosin molecules in the transition state of ATP hydrolysis and 'primed' to execute a power stroke. In this state the 'back door' is closed, helping to explain why P_i is bound so tightly to M·ADP·P_i.

Assuming that the motor domain of myosin binds to actin the same way as it does in the near rigor state, the bent shape positions the light chain domain markedly towards the pointed end of actin (upwards in figure 2*b*) as expected for a pre-power-stroke state. The tilting of the light chain region enables it to serve as a lever arm to magnify small atomic-scale motions at the nucleotide-binding site into the 5–10 nm motion required to slide the filaments (Irving *et al.* 1992; 1995; Rayment *et al.* 1993*a*). Coupling between the position of switch II and the lever arm is through the α-helix following switch II, a conserved phenylalanine that kinks and twists this helix, and the segment of the heavy chain just before the light chain domain, termed the converter (Geeves & Holmes 1999).

As in other ATPases and GTPases, the presence of either the γ-phosphate of ATP or product P_i with ADP pulls switch II into the closed position, and when P_i is absent switch II moves away (Vale 1996). A closed switch II is associated with the lever arm 'up' in the primed position and an open switch II with the lever arm 'down'. Thus the two structures give a reasonable hypothetical depiction of the structural changes that cause the filament to slide and how they are coupled to P_i release (Holmes 1997). But

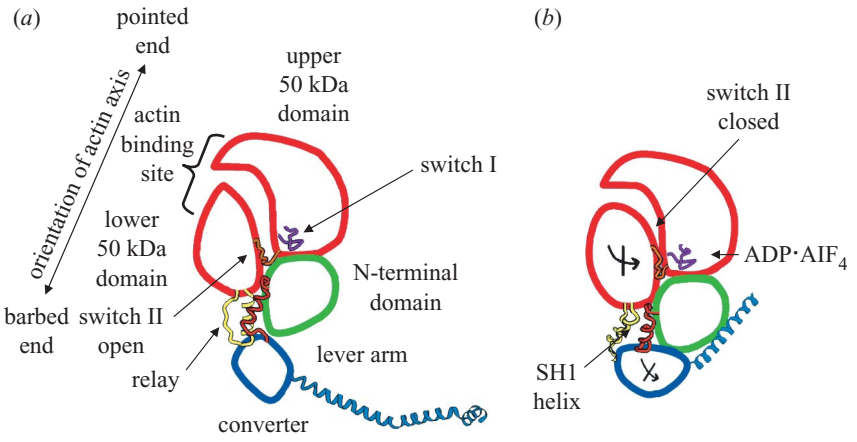


Figure 2. Schematic representations of myosin heads in (*a*) the near rigour state (Rayment *et al.* 1993*b*) and (*b*) the pre-power-stroke state (Fisher *et al.* 1995; Dominguez *et al.* 1998). Subdomains in the motor domain are shown as compact globular elements connected by joints. The light chains have been removed from the lever arm for clarity. (Adapted from Houdusse *et al.* (2000).)

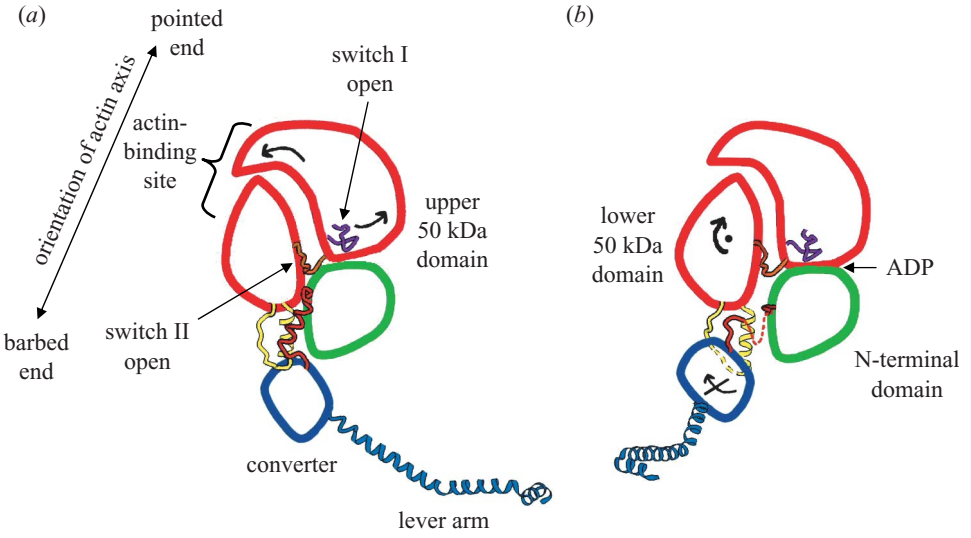


Figure 3. Schematic representations of myosin heads in (*a*) the presumed strongly bound rigour state (Coureux *et al.* 2003; Reubold *et al.* 2003) and (*b*) the presumed detached state (Houdusse *et al.* 2000).

which motion comes first? If the transition to the force generating state precedes P_i release, as argued in § 4, then how does actin trigger the structural change while P_i is holding switch II in place? This difficulty led several authors to suggest that P_i release 'prompts' the switch II structural change, the reverse of Scheme 1 (Houdusse & Sweeney 2001; Spudich 2001). However that idea causes a different problem: how can P_i dissociate when the back door is closed?

Crystal structures of myosin in the presence of ADP and beryllium fluoride illustrate a general point about predicting dynamics from structural snapshots. In some of the ADP·BeF_3 structures, switch II is closed (figure 2*b*, lever up) (smooth muscle myosin (Dominguez *et al.* 1998) and *Dictyostelium* myosin II (Reubold *et al.* 2003)), but it can be also be open (figure 2*a*, lever down, *Dictyostelium* myosin II (Fisher *et al.* 1995)). Thus myosin can adopt either of these conformations in nominally the same state of bound ligands and there is no information given from these structures whether the motion precedes or follows the dissociation of the P_i analogue. These and other considerations suggested that further conformations of the myosin head would be found (Cooke 1997; Goldman 1998; Geeves & Holmes 1999).

6. RESURRECTION OF SCHEME 1: STRUCTURAL CHANGES

Two further distinct crystal forms of the myosin head have indeed been discovered. In *Dictyostelium* myosin II (Reubold *et al.* 2003) and in myosin V (Coureux *et al.* 2003) in the absence of nucleotide, the cleft between the upper and lower 50 kDa domains is tightly closed (figure 3*a*), suggesting that it is very close to the strongly actin-bound form at the end of the power stroke. Switch I, another conserved loop at the active site that can form a salt bridge with switch II and hydrogen bonds to the γ-P_i and to the nucleotide-associated magnesium ion, has moved aside, breaking these interactions. Closure of the actin-

binding cleft in the 50 kDa domain opens switch I (curved arrows in figure 3*a*), decreasing interactions with the nucleotide and thus explaining the reciprocal affinity myosin has for nucleotides and actin. This coupling is especially prominent in fast myosins such as skeletal muscle (Cremo & Geeves 1998).

Other studies, using fluorescent probes flanking the 50 kDa cleft (Yengo *et al.* 2002; Conibear *et al.* 2003) and high-resolution cryo-electron microscopic maps of myosin-decorated actin (Holmes *et al.* 2003), confirmed that the cleft closes tightly when myosin binds strongly to actin and that this closure opens switch I. Kinesin and GTP-binding proteins have switch I in this open position (Vale 1996), but the open position of switch I had not been observed previously in myosin crystal structures. A study of binding of ATP analogues with large groups in place of the γ-P_i, though, had predicted its appearance (Pate *et al.* 1997).

The other major conformation of myosin solved so far was found in scallop myosin complexed to ADP (Houdusse *et al.* 1999, 2000). In this structure, the sections of the motor domain are coupled very loosely to each other and the lever arm is tilted downwards, far beyond the rigor position (figure 3*b*). It is likely to represent a conformation of myosin detached from actin or else ready to detach after being dragged beyond the end of the working stroke by the action of other crossbridges in rapidly shortening muscle (Cooke 1999).

These new conformations and independent motions, switch I coupled to the actin-binding cleft and switch II coupled to the lever arm, allow more flexibility in models of the structural changes associated with the biochemical and mechanical steps of the actomyosin cycle. Opening of switch I provides an exit route for product P_i as an alternative to the 'back door'. Actin binding to AM·ADP·P_i may trigger the power stroke via closure of the 50 kDa cleft, opening switch I, thereby reducing its interactions with switch II, and allowing switch II to open. In this scenario, P_i dissociates after these steps.

Alternatively, the initial generation of force may be the weak to strong transition of AM·ADP·P_i to AM′·ADP·P_i, rotating the whole myosin head before the internal structural change of switch II motion coupled to the lever arm (Ostap *et al.* 1995; Taylor *et al.* 1999; Tsaturyan *et al.* 1999). The working stroke would then take place in two steps (Taylor *et al.* 1999): rotation of the whole head followed by the lever arm swing associated with P_i release. In this scheme, P_i could dissociate either before or after switch II opens. Such a two-step mechanism for energy transduction would raise efficiency, maintain high tension through much of the working stroke, and produce a flat 'T_2 curve' (Piazzesi *et al.* 1995).

7. MYOFIBRILS AND SINGLE MOLECULES

If dissociation of P_i from AM′·ADP·P_i takes place at only 75 s^{-1}, as suggested by the experiments mentioned earlier on acto-S1 with the phosphate-binding protein (White *et al.* 1997), and ADP release and ATP binding are much faster, then AM′·ADP·P_i may be significantly populated during an active contraction. Lionne *et al.* (1995, 2002) found that the predominant steady-state intermediate contains P_i in actively shortening myofibrils and in activated myofibrils prevented from shortening by moderate cross-linking of the

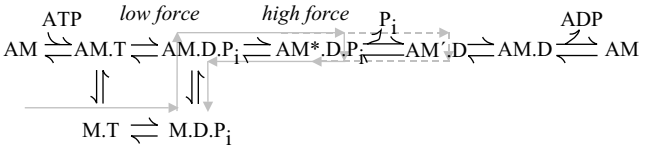


Figure 4. Kinetic trajectories describing single actomyosin interactions with two differing dynamic loads of the isometric clamp. In both trajectories, the transition to a force generating state occurs before P_i release. At a high dynamic load (solid line), force is applied by the laser tweezers quickly, before P_i release, so that reversal of force production does not require P_i binding. At a moderate dynamic load (dashed line), force is applied more slowly, allowing time for P_i dissociation, and requires its rebinding before reversal and detachment.

two sets of filaments, These preparations mimic rapidly shortening and isometric muscle fibres, respectively, but they eliminate diffusion artefacts and enable transient perturbations by rapid mixing. The results suggest that the force-generating intermediate is AM′·ADP·P_i.

Pate & Cooke (1989) argued that the steady-state inhibition of isometric tension by P_i in the filament lattice should be logarithmic if P_i release accompanies force generation in one step. However, if Scheme 1 applies, at very high P_i concentration the force inhibition plateaus at the level set by the equilibrium between AM·ADP·P_i and AM′·ADP·P_i. Tesi *et al.* (2000, 2002) tested the P_i dependence of force developed by isometrically held individual myofibrils or bundles of a few myofibrils over a range of P_i concentrations from *ca*. 5 μM to 70 mM. Without diffusion artefacts, the P_i concentration within the contracting preparation is the same as in the bathing solution. At 80 mM P_i, the force data from psoas myofibrils deviate slightly from the logarithmic relationship fit to the force values at lower P_i concentrations. Their interpretation of this observation is the same as Scheme 1, but it is difficult to raise the P_i concentration high enough to show a clear plateau. In soleus myofibrils, force plateaus at a higher level, more strongly supporting force generation by AM′·ADP·P_i (Tesi *et al.* 2002).

For single-molecule actomyosin interactions with myosin II, the 'three-bead assay' (Finer *et al.* 1994) has been widely adopted (Molloy *et al.* 1995; Guilford *et al.* 1997; Steffan *et al.* 2003). In this technique an actin filament is suspended between two optically trapped polymer beads and myosin is sparsely bound to pedestals on the microscope slide. When the actin is brought into contact with the myosin, active interactions are monitored by displacement of the beads. We developed a single-molecule isometric force clamp, which uses feedback in the three-bead assay to restore the position of an actin filament to its preset position within a finite time, in response to myosin interactions (Takagi *et al.* 2000). By changing the feedback gain, the response time, τ_r, of the force clamp, and therefore the dynamic load on actomyosin, can be adjusted. With high feedback settings, giving a stiff dynamic load ($\tau_r = 1$ ms), the average duration of isometric actomyosin events of whole rabbit skeletal myosin was only 10 ms and virtually independent of ATP and P_i concentrations (Takagi *et al.* 2004). Moreover, these events were much shorter than durations measured without feedback (Takagi *et al.* 2002).

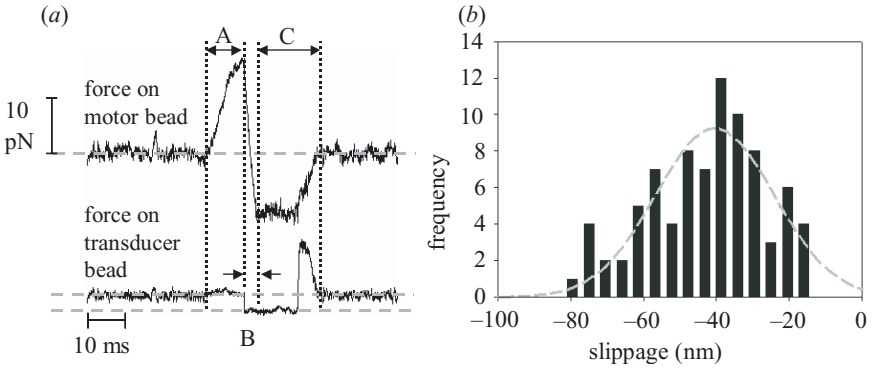


Figure 5. (*a*) An example of a 'bipolar' actomyosin event at very low (1 nM) ATP concentration. An initial large positive force deflection (phase A) is followed by a sudden force reversal (phase B). During the force reversal, the motor trap (upper trace) and the transducer bead force (lower trace) show that the myosin abruptly 'slipped' to a different position. The value of negative force is limited by the pre-tension (11 pN) applied to actin. At this limit, the actin becomes slack (phase C). At the end of the event, the forces return to their unattached signal levels. (*b*) The mean slippage of myosin backwards along actin was *ca*. 40 nm from a Gaussian fit (dashed line) to the histogram of transducer bead displacements (bars).

These results suggest that the attachment events end without completing the ATPase cycle.

Figure 4 shows a scheme for the ATPase reaction on which possible detachment paths are highlighted. At moderate dynamic load ($\tau_r = 10$ ms), the event durations (20–40 ms) were intermediate between the high dynamic load and no feedback. Unlike those at a stiff dynamic load ($\tau_r = 1$ ms), they depended on both ATP concentration (24.9 and 47.8 ms for 10 and 1 μM, respectively) and P_i concentration (25.2 and 47.8 ms for 10 mM and less than 2 μM, respectively; [ATP] = 1 μM). These results suggest that mechanical work by actomyosin can be reversed by an applied load and they strongly indicate that force generation in individual actomyosin interactions occurs before P_i release. The non-cycling detachment pathway requires P_i binding only if the interactions last 20–40 ms (dashed line) implying that AM′·ADP·P_i generates force as in Scheme 1.

Figure 5*a* shows a surprising characteristic of *ca*. 10% of actomyosin interactions observed in the isometric optical clamp when ATP concentration was reduced to *ca*. 1 nM, so that most of the interactions between myosin and actin occur without any bound nucleotide. Following the development of tension to *ca*. 15 pN, the force rapidly reverses and saturates the feedback system as the actin on one side of the myosin molecule goes slack. The amount of sliding produced upon this force reversal varies between 16 and 70 nm, averaging 35 nm (figure 5*b*). Whether this behaviour represents the second head of a myosin molecule attaching towards the barbed end of actin under these high load conditions, or possibly folding back of the myosin rod to another position on the substrate will require further experiments.

8. CONCLUSIONS

These data are all consistent with the order of events suggested before: M·ADP·P_i binds weakly, then strongly to actin, generating force. Then P_i dissociates, possibly further increasing force. Structural and kinetic considerations that challenged this order of events have been supplemented with further data that seem to rationalize it again. If the initial weak-to-strong transition within actomyosin produces a force-generating state, then P_i release could take place before or after the active site change (opening of the switch region or twisting of the core β-sheet) that causes rotation of the lever arm. The detailed sequence of motions that trigger force generation upon binding of M·ADP·P_i to actin may be different from the priming of the working stroke that takes place just before myosin hydrolyses ATP. The exact sequence is thus unlikely to be revealed until crystal structures of actomyosin are solved. Meanwhile, coupling of switch I to the actin binding cleft and switch II to the lever arm provide plausible routes for this allostery.

Note added in proof. D. A. Smith and J. Sleep have recently published work (*Biophys. J.* 2004 **87**:442–456) that compares models of tension recovery after quick length changes in muscle, which supports the same conclusion as given here that P_i release follows force generation.

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GLOSSARY

ADP: adenosine 5′-diphosphate
AM: actomyosin
ATP: adenosine 5′-triphosphate
GTP: guanosine 5′-triphosphate
M: myosin
P_i: orthophosphate
S1: myosin subfragment 1