Using optical tweezers to relate the chemical and mechanical cross-bridge cycles

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In most current models of muscle contraction there are two translational steps, the working stroke, whereby an attached myosin cross-bridge moves relative to the actin filament, and the repriming step, in which the cross-bridge returns to its original orientation. The development of single molecule methods has allowed a more detailed investigation of the relationship of these mechanical steps to the underlying biochemistry. In the normal adenosine triphosphate cycle, myosin-adenosine diphosphate-phosphate ($M \cdot ADP \cdot P_i$) binds to actin and moves it by *ca.* 5 nm on average before the formation of the end product, the rigor actomyosin state. All the other product-like intermediate states tested were found to give no net movement indicating that $M \cdot ADP \cdot P_i$ alone binds in a pre-force state.

Myosin states with bound, unhydrolysed nucleoside triphosphates also give no net movement, indicating that these must also bind in a post-force conformation and that the repriming, post- to pre-transition during the forward cycle must take place while the myosin is dissociated from actin. These observations fit in well with the structural model in which the working stroke is aligned to the opening of the switch 2 element of the ATPase site.

Keywords: myosin; actin; single molecule; cross-bridge

1. CORRELATIONS BETWEEN BIOCHEMICAL AND MECHANICAL STATES

The Lymn & Taylor (1971) model (figure 1) remains a useful summary of our state of knowledge of the coupling of the biochemical actomyosin ATPase cycle with the mechanical cross-bridge cycle. The key event of the cycle is the binding of actin to an intermediate myosin state $M \cdot ADP \cdot P_i$, which results in the acceleration of product release and the working stroke, but for further cycles to take place there must also be a repriming step in which the cross-bridge reverts to its original orientation. These two important mechanical steps are linked by the two simple steps of actin binding and dissociation. In this paper, we review the application of single molecule experiments to the problem of associating the mechanical steps more exactly with the biochemical steps.

The general antagonistic effect of ATP and actin binding to myosin has been known almost since the time the two proteins were separated, but it was not until the tight binding constants for ATP (Goody *et al.* 1977; Cardon & Boyer 1978) and actin (Marston & Weber 1975) were measured that a full appreciation of this effect was possible. It became clear that about half the free energy change of ATP hydrolysis was associated with P_i release, or more precisely, with that part of the scheme between the $M \cdot ADP \cdot P_i$ binding to actin and the equilibrium ternary $AM \cdot ADP$ complex (White & Taylor 1976). The cross-bridge throw

takes place in this part of the cycle and must be driven by this free energy difference. However, the free energy change upon M alone binding to actin is even greater than upon $M \cdot ADP \cdot P_i$ binding to actin, and it is important to determine whether this energy can be coupled to a working stroke. This question relates directly to the '3G' model of muscle contraction (Geeves *et al.* 1984), which was based on the observation that all myosin intermediates bind to actin in two steps. In the first, an intermediate is formed in which actin is bound weakly and it is only in the subsequent step that a strong state is formed. This idea fitted in well with the earlier observations of Eisenberg & Greene (1980), who had suggested dividing myosin intermediates into two classes based on their strength of binding to actin. The weak class consisted of $M \cdot ATP$ and $M \cdot ADP \cdot P_i$ and the strong class $M \cdot ADP$ and M . This division was supported by their observation that the binding of the strongly bound intermediates to thin filaments was regulated by Ca^{2+} , whereas the binding of the weakly bound intermediates was unaffected, suggesting that the two classes of myosin intermediate represented distinct conformations (Chalovich *et al.* 1981). It was natural to suppose that the transition from the weak to the strong state corresponded to the working stroke. If Eisenberg's weak to strong transition corresponded to Geeves's transition between the initial and final actin bound states, then one would expect to observe a displacement of the actin filament when myosin either with no bound ligand or with bound ADP, PP_i , or AMP PNP, attaches to actin. These ideas can be addressed directly at the single molecule level because all the different myosin intermediate states can be allowed to

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Figure 1. Lymn-Taylor model of ATPase cycle. States in italics represent post-force states.

react repeatedly with actin in a way that is not possible for a larger scale system such as a single muscle fibre.

In the Lymn-Taylor scheme, the repriming step was associated with the hydrolysis step for two reasons. First, in this simple scheme with a minimal number of states there was very little choice. Second, it avoided any problem of what is essentially a reversal of the force-generating step occurring while actin is associated. However, Eisenberg and Greene presented a convincing case for an alternative location. As argued above, the two weak states $M \cdot \text{ATP}$ and $M \cdot \text{ADP} \cdot \text{P}_i$ should be in the same conformation and thus if $M \cdot \text{ADP} \cdot \text{P}_i$ is in a pre-force state so as to allow a movement of the cross-bridge upon binding to actin, then so must $M \cdot \text{ATP}$. In this case the repriming step would have to take place between two $AM \cdot \text{ATP}$ states (see figure 2). They also pointed out that this reversal of the working stroke while attached was not a problem because the rate of detachment of actin from $AM \cdot \text{ATP}$ is three orders of magnitude greater than the fibre ATPase rate and thus the time-averaged force is little affected. Recent experiments have revealed the existence of a second $M \cdot \text{ATP}$ state (Urbanke & Wray 2001), which appears to be in a post-force conformation. The force of Eisenberg and Greene's argument has thus been weakened but the need to directly determine whether repriming occurs before or after actin dissociation remains. Unfortunately, the location of the repriming stroke cannot be dealt with as directly as that of the working stroke and we have addressed the problem by studying the reversal of the reaction.

2. THE BINDING OF MYOSIN-NUCLEOTIDE COMPLEXES TO ACTIN

This topic has been introduced here in a historical context but the results fit well with the model derived from recent crystallographic, electron microscopic and fluorescence studies, and they will be discussed directly in terms of this model. Many of the structural elements of the ATPase site are characteristic of P-loop proteins, and the opening of the switch 1 and switch 2 elements are associated, respectively, with the development of a tight actin binding site and the occurrence of the cross-bridge throw (Geeves & Holmes 1999; Holmes *et al.* 2003). Studies of the fluorescence states of both native rabbit myosin S1 (Urbanke & Wray 2001) and of *Dictostylium* myosin heads engineered with tryptophans at specific locations (Wakelin *et al.* 2002) extended these ideas by allowing the provisional characterization in terms of pre- or post-force conformations of the various myosin intermediates (see figure 3). The finding that $M \cdot \text{ATP}$ exists in two conformational states in approximately equal concentrations (Malnasi-Csizmadia *et al.* 2001), one ($M_o \cdot \text{ATP}$) with an open and the other ($M_c \cdot \text{ATP}$) a closed switch 2 was of particular importance, as crystallographers had appreciated that switch 2 had to be

Figure 2. Eisenberg and Greene model. States in italics represent post-force states.

closed to allow catalysis of the hydrolysis step. The observations were in accord with the innovative studies of Suzuki *et al.* (1998), who used fluorescence resonance energy transfer to monitor lever arm movement and relate it the myosin-bound nucleotide state.

3. SINGLE MYOSIN MOLECULE MECHANICS

The first genuinely single molecule studies of the myosin motor were made by holding a fluorescently labelled actin filament taut between two optically trapped beads (Finer *et al.* 1994) and presenting the actin to a myosin molecule attached to a third bead fixed to the surface of the cover slip. This approach has proved more convenient than the pioneering methods of Yanagida and colleagues using microneedles (Kishino & Yanagida 1988) and this dumbbell method is now used in many laboratories (Molloy *et al.* 1995a; Guilford *et al.* 1997; Steffen *et al.* 2001). The first major development of the method was by Molloy's group, who realized that Brownian motion allowed several actin monomers to interact with the myosin and that the distribution of positions of the dumbbell during myosin-bound periods mirrored the distribution while detached from myosin but was displaced by the throw of the cross-bridge. Moreover, the reduction in Brownian motion of the dumbbell upon attachment of myosin could be used to detect the occurrence of interactions with myosin (Molloy *et al.* 1995a). A brief period of recording with a particularly weak trap strength is shown in figure 4a and it can be seen that the positions of bound interactions range over more than 50 nm because of the Brownian motion of the free dumbbell. By contrast, when (for the same dumbbell) the stiffness of the traps was increased by a factor of over 20 times, the direction of the working stroke (negative in this case) becomes clear even from this short record (figure 4b). Because the first use of the variance record to detect events, the method has been refined by several laboratories (e.g. Mehta *et al.* 1997; Veigel *et al.* 1998) and the compliance of all the components in the system can be determined (Smith *et al.* 2001). Simplistically, the motion of the dumbbell is limited by the combined stiffness of the two traps when the actin is dissociated from actin and by the stiffness of the myosin when associated. However, in practice, when the myosin is bound, thermal noise is limited by the stiffness of the myosin and of the bead actin linkages in series.

As the two trapped beads are nominally spherical, it might be expected that the dumbbell would be free to rotate about its axis. Molloy *et al.* (1995b) had presented preliminary evidence that this might not be the case, which encouraged us to investigate the matter further. By correcting the drift of the objective with respect to the microscope stage, we were able to see both the 37 nm repeat of the actin helix and the 5.5 nm monomer

Figure 3. ActoS1 ATPase mechanism deduced from crystallographic and fluorescence studies. The subscript o refers to the state of the switch 2 element. $\text{AM}_0 \cdot \text{ATP}$ is in grey to signify that it may not be a significant part of the reaction pathway.

Figure 4. Records in the presence of ATP. (a) With a very weak trap (combined trap stiffness $0.0067 \text{ pN nm}^{-1}$) the trace is broad and events occur throughout the noise band. (b) The same dumbbell with much stiffer traps (0.16 pN nm^{-1}) showing the bound events largely outside the noise band as a result of the size of the working stroke (6 nm in this record), relative to the standard deviation of the free dumbbell (5 nm).

repeat. An example is shown in figure 6b. The ability to see these repeats opens up several possibilities for the future but it has an immediate value for the purposes of quality control. If more than one myosin head is interacting with the actin it is likely that the quality of marking of the monomer repeat will be reduced and such experiments can be rejected.

Unfortunately, the location of the repriming stroke cannot be determined directly during hydrolysis of MgATP because most interactions of actin with myosin are associated with product release and represent the working stroke. For skeletal myosin, the kinetics are such that a single molecule record of actomyosin interactions in the presence of MgATP consists of a series of transitions between the free state associated with a high variance of bead position, and the bound state in which actin is associated with myosin in a post-force position, associated with a low variance. The repriming step is included in the partial reaction in going from the bound to the free state but the associated position change is simply a reversal of the cross-bridge throw. If the time resolution of the experiment were adequate to reveal the elementary steps, the chemical nature of the repriming step could be determined by observation of the cross-bridge cycle in the presence of ATP as shown in figure 5. In the schematic for the Lymn–Taylor model (figure 5a), the rigor state binds ATP and at the end of the second grey band actin dissociates to form $\text{M} \cdot \text{T}$. Repriming is associated with the hydrolysis step, but as it takes place while actin is dissociated it leaves no mark on the record of bead position. By contrast, for the Eisenberg model, the rigor state binds ATP and this is followed by the repriming step between two $\text{AM} \cdot \text{T}$ states. The actin

Figure 5. Simulated plots of bead position as a function of time, predicted in terms of (a) Lymn–Taylor and (b) Eisenberg–Greene models. The grey bands represent the transitions between periods in which the dumbbell is free and those in which it is bound in the rigor state. The position of the dumbbell cannot be resolved during these periods. Arrows mark the times of the repriming steps in the two schemes. For the Eisenberg–Greene scheme, repriming occurs at the transition between two $\text{AM} \cdot \text{T}$ states, whereas for the Lymn–Taylor scheme, it occurs at the transition between $\text{M} \cdot \text{T}$ and $\text{M} \cdot \text{D} \cdot \text{P}$. In both schemes the latter transition is marked by a vertical line.

is still bound during this process and a step is recorded (figure 5b). Unfortunately, the time resolution is not adequate to characterize the position during the grey-banded period and a less direct method has to be employed.

The question of which is the dominant pathway can be addressed by investigating the reverse reaction because detailed balance requires that the relative fluxes of parallel pathways are the same in the forward and reverse directions. As mentioned above, during ATP turnover the change in dumbbell position during the transition from AM to $\text{M} \cdot \text{ADP} \cdot \text{P}_i$ is dictated by the position of AM , which was established during product release and which does not characterize the repriming step. To measure any stroke associated with repriming it is necessary to follow the transition from $\text{M} \cdot \text{ATP}$ directly to $\text{AM} + \text{ATP}$. During acto-S1 ATPase activity, *ca.* 10% of interactions of actin with the dissociated myosin intermediates ($\text{M} \cdot \text{ATP} \leftrightarrow \text{M} \cdot \text{ADP} \cdot \text{P}_i$) result in ATP dissociation (Sleep & Hutton 1978), but it is not possible to associate a given interaction with ATP or product release. To overcome this problem, a particular class of analogues of ATP (NTP) has been employed: these analogues share the property that the rate-limiting step of myosin NTPase is the hydrolytic step so that the dominant steady-state intermediate is $\text{M} \cdot \text{NTP}$. To validate this approach it was necessary to confirm that the bound state was the rigor AM state and this could be addressed fairly unequivocally by observing that the duration of bound events was inversely proportional to the $[\text{NTP}]$. It was also necessary to show that the dominant

form of interaction with actin resulted in NTP rather than product release and again the kinetics of the process indicated that this was correct.

The term working stroke is commonly used both for the observed net displacement observed in a trap experiment during ATP turnover and for the distance moved in the hypothetical conformational change (h in Huxley & Simmons 1971). In this paper, the term working stroke is used as a general term to describe the cross-bridge movement associated with the steps between $M \cdot ADP \cdot P_i$ and AM. The term mean displacement of the dumbbell under specified conditions has been used for the experimentally observed difference in the means of the Gaussian distributions describing the position of the dumbbell when free and when bound to actin (Molloy *et al.* 1995a). In our experiments the dumbbell is moved an integral number of actin repeats (usually $4 \times$ or 5×37 nm) past the myosin so as to average out the helical structure of the actin (Smith *et al.* 2001).

4. THE BINDING OF PRODUCT-LIKE STATES TO ACTIN

The working stroke is generally regarded as resulting from the switch 2 closed, pre-force $M_c \cdot ADP \cdot P_i$ state binding to actin and undergoing the transition to the switch 2 open, post-force $AM_o \cdot ADP$ state. On this basis, dumbbell displacements will be observed only in trap experiments when myosin states in the closed form bind to actin (reviewed by Geeves & Holmes 1999). For example, if $M \cdot AMP$ PNP were in the switch 2 closed, pre-force conformation, one would expect a power stroke from the sequence of reactions in equation (4.1). Myosin binds AMP PNP, PP_i and ADP very much more tightly than actomyosin so that a concentration can readily be chosen which is sufficient to saturate the myosin while still being low enough for it to be dissociated from AM for most of the time.

A critical requirement for measurement of the displacement is the recording of a sufficient number of events and both the binding and dissociation rates need to be in a convenient range. For acto-S1 in solution the rate of binding is faster for intermediates later in the cycle than for the $M \cdot ADP \cdot P_i$ state monitored during ATPase activity. This prediction was borne out in that, with both PP_i and AMP PNP, an additional 75 mM KCl was required to reduce the rate of binding and allow well-defined single events to be resolved (Steffen *et al.* 2003). The greater problem proved to be achieving an adequately high dissociation rate and this was greatest for myosin in the absence of any ligand. A high ionic strength was required (0.5 M KCl) and even under these conditions, long (400 s) sampling periods were necessary to acquire sufficient events.

Figure 6a shows a record in the presence of PP_i and it can be seen that the general appearance is very similar to that with ATP (figure 4a,b). In both cases, the myosin intermediate during the bound period is basically the AM rigor state, as evidenced by the plots of the rate of dissociation against ligand concentration in figure 6c. It was only in the case of ADP that the binding constant was sufficiently tight

Figure 6. (a) Experimental record in the presence of 200 μM PP_i . (b) Events recorded in the presence of 500 μM PP_i . The grey band indicates the motion of the dumbbell during the period of data collection, the width corresponding to $\pm$ s.d. of the free dumbbell position. The ability to assign events to specific monomers with PP_i is clearly evident. (c) Dependence of the dissociation rate on $[PP_i]$ indicating that the bound state is AM.

(ca. $10^4 M^{-1}$) to allow experiments at near saturating ligand concentration. As expected, the rate of dissociation approached a saturation value indicating that the bound state was primarily AM·ADP. At low [ADP] the experiment reports on the displacement arising from the reaction $M \cdot ADP \rightarrow AM$ and at high [ADP] from $M \cdot ADP \rightarrow AM \cdot ADP$. As no significant displacement was observed under either circumstance we were able to eliminate the possibility that there were equal and opposite displacements in forming AM·ADP and in the subsequent dissociation of ADP. As expected, the stiffness of the myosin during the bound periods (greater than 1 pN nm⁻¹) is similar for the product-like states and for ATP, consistent with AM (or AM·ADP) being the relevant state under all circumstances. The binding events could also be assigned to individual actin monomers just as in the case for ATP (see figure 6b).

Table 1. Summary of working strokes observed when product-like myosin intermediates bind to actin.

nucleotide (μM)	ATP	PP_i 500	AMP-PNP 1000	ADP 50	none
working stroke (nm)	5.4	0.4 ± 1	0.1 ± 0.2	0.6 ± 0.5	0.1 ± 0.9

Table 1 summarizes the data for the binding of M , $\text{M} \cdot \text{ADP}$, $\text{M} \cdot \text{PP}_i$ and $\text{M} \cdot \text{AMP PNP}$ to actin. It is evident that the results fall very much in line with expectations. The displacement recorded was within experimental error of zero for all cases, the only product state we found to give a displacement was the $\text{M} \cdot \text{ADP} \cdot \text{P}_i$ state that binds during the normal ATPase cycle. This result implies $\text{M} \cdot \text{ADP} \cdot \text{P}_i$ alone is in a conformational pre-force state, which is distinct from the post-force conformation of the final rigor AM state. All the other product states appear to already be in the post-force rigor conformation at the time of binding. These observations are not in accord with the basic 3G model of muscle contraction in which all myosin intermediate states bind initially in a pre-force form (Geeves *et al.* 1984).

It is important to appreciate when considering the significance of any displacements of dumbbell position observed upon myosin intermediates binding to actin, that the method does not assay the ratio of concentrations of the pre- and post-force forms of the intermediate ($[\text{M}_c]/[\text{M}_o]$) but the ratio of concentrations times the rates of binding ($(k_c[\text{M}_c]/k_o[\text{M}_o])$, where k_c and k_o are the rates of binding of the closed and open forms, respectively. By contrast, related studies in which the muscle fibre X-ray diffraction pattern is compared in the presence of different ligands do appear to provide a measure of the relative concentrations of the open and closed forms (Xu *et al.* 2003). Tryptophan fluorescence studies indicate that $\text{M} \cdot \text{AMP PNP}$ exists in an approximately 50/50 mixture but we still observed no detectable displacement. We interpret this observation in terms of the switch 2 open states tending to bind more rapidly to actin than the switch 2 closed states. Fluorescence studies indicate that *ca.* 10% of the $\text{M} \cdot \text{PP}_i$ intermediate is in the pre-force form, so that even if the two forms bound at similar rates, a displacement would be barely detectable. The potential significance of open switch 2 forms of intermediates binding more rapidly than closed forms is considered in the conclusion.

5. THE REPRIMING STEP

Following Lymn & Taylor (1971), the obvious pathway of dissociation of actomyosin by ATP is marked in black in figure 3 but the grey pathway through $\text{AM}_c \cdot \text{ATP}$ is, in principle, equally viable and the operative one needs to be determined. To this end, we have used single-molecule methods to analyse the reaction $\text{A} + \text{M} \cdot \text{T} \rightarrow \text{AM} \cdot \text{T} \rightarrow \text{AM} + \text{T}$. A group of ATP analogues (GTP, ITP and $\text{ATP}\gamma\text{S}$) was employed, which, unlike ATP, give rise to $\text{M} \cdot \text{NTP}$ as the dominant steady-state intermediate in NTPase activity. The K_m of myosin for each of these analogues is higher than for ATP but still very low (less than $1 \mu\text{M}$) so that the myosin was saturated with the analogue throughout the concentration range used. The first step in the reaction path under investigation is the formation of the $\text{AM} \cdot \text{NTP}$ state but the lifetime of this state is controlled by the fast rate of dissociation and is too short to be detected in

our optical trap experiments. The expectation is that only the AM state will be detected and the observations of the exponential dependence of the number of events against event lifetime and of the linear dependence of the rate of dissociation upon analogue concentration provide confirmation of this. AM is the only significant state present in this range of NTP concentrations. The records of bead position with the analogues look similar to those with ATP in that the variance during bound periods is typically more than 12 times less than during free periods and the bound periods are characterized by the periodic character of the actin helix. The state AM has an open switch 2 element and we must assume that the next state is $\text{AM}_o \cdot \text{T}$. Correspondingly, everything must reverse through this state, which acts as the defining junction point between two fluxes (see figure 3). If the preceding state were $\text{M}_o \cdot \text{T}$, no working stroke would be observed (Lymn & Taylor 1971), whereas if the preceding state were $\text{AM}_c \cdot \text{T}$, a working stroke would be observed (Eisenberg & Greene 1980). The relative fluxes from these two sources are controlled by the rates of all the elements in the square $\text{AM}_o \cdot \text{T}$, $\text{AM}_c \cdot \text{T}$, $\text{M}_o \cdot \text{T}$ and $\text{M}_c \cdot \text{T}$ (see figure 3).

Actin is a poor activator of the myosin NTPase because the actin-accelerated step, product release, is no longer the major rate-limiting step and furthermore the species with which actin interacts to accelerate product release is now only a small fraction of the total myosin. The rate of actin activated hydrolysis of GTP is some 40 times less than for ATP (10°C , low ionic strength; White *et al.* 1993). We observed that in the optical trap the interaction rate, far from being much lower, was in fact significantly higher with GTP, ITP and $\text{ATP}\gamma\text{S}$ than with ATP (Steffen & Sleep 2004; see table 2). For dumbbell experiments, the duration of events is strictly controlled by the second-order rate of NTP binding but the rate of observing events is critically dependent on the exact position of the dumbbell with respect to the fixed myosin bearing bead, thus a relatively large number of experiments are needed to make a comparison. Table 2 includes the rate of observed events for the three analogues relative to that for ATP and it can be seen that the rate is substantially faster and this observation indicates that the reaction being observed is $\text{M} \cdot \text{NTP} + \text{A} \rightarrow \text{AM} \cdot \text{NTP} \rightarrow \text{AM} + \text{NTP}$. It had previously been shown that the addition of actin to an equilibrium mixture of $\text{M} \cdot \text{ATP}$ and $\text{M} \cdot \text{ADP} \cdot \text{P}_i$ releases *ca.* 10% of the bound nucleotide as ATP (Sleep & Hutton 1978). A similar study showed that in the case of GTP, greater than 90% of the bound nucleotide is released as GTP (White *et al.* 2004), thus confirming conclusions about the nature of the observed interactions.

The displacements of the dumbbell observed in the presence of NTP are shown in table 2 (Steffen & Sleep 2004). In each case, the value is not significantly different from zero and thus we conclude that $\text{M} \cdot \text{GTP}$, $\text{M} \cdot \text{ITP}$ and $\text{M} \cdot \text{ATP}\gamma\text{S}$ all bind to actin in the same conformational state as the final AM rigor state. If this observation of a lack

Table 2. Summary of working strokes observed when ATP-like myosin intermediates bind to actin.

nucleotide	ATP	ITP	GTP	ATP γ S
working stroke (nm)	5.4	0.8 $\pm$ 0.6	0.6 $\pm$ 0.7	0.2 $\pm$ 0.3
on rate ^a	1 $\times$	5.2 $\times$	4 $\times$	4 $\times$

^a Frequency of events relative to that in the presence of ATP.

of a displacement is to be significant, evidence is required that these ATP analogues do give a displacement upon product release. However, just as in the case of ATP it is difficult to get information about repriming because most of the interactions are associated with product release, in the case of the analogues it is not possible to gain evidence for a displacement associated with product release because most of the events are associated with ATP release. The main evidence for a normal cross-bridge throw comes from observations in fibres. In the presence of ITP, the tension in skinned fibres is *ca.* 35% of that with ATP and the shortening velocity is *ca.* 20% (Burton *et al.* 2004). In view of the slow turnover rate, it seems likely that a normal cross-bridge throw is associated with product release. There is no conflict between the observed tension in a fibre and our inability to see displacements associated with product release because the fibre experiments are carried out at near-saturating concentrations of ITP, which greatly reduces the lifetime of AM states but does not affect the lifetime of the tension-bearing, AM $\cdot$ product states. By definition, at saturation the concentration of the rigor AM state is zero.

A fairly convincing case can thus be made in the case of the analogues for the repriming step taking place while myosin is dissociated but the question of whether it is safe to extrapolate the conclusion to the case of ATP needs to be considered. A possible reason for the low observed rate of the hydrolytic step for the analogues is that the preceding step (the open to closed transition) is unfavourable. If this were the case it would also increase the ratio of open state binding relative to closed. Fortunately, Wakelin *et al.* (2002) reported that the equilibria between the open and closed forms of M $\cdot$ NTP for the analogues are all quite comparable to those for ATP (*ca.* 0.4, $\leq$ 0.3, $\leq$ 0.7 and $\leq$ 1.7 for ATP, ATP γ S, GTP and ITP, respectively). In the case of ATP, the known kinetics favour dissociation before repriming. The rigor state AM is an open state (AM_o) and the first ATP state must be AM_o $\cdot$ T. The first-order rate of actin dissociation from AM $\cdot$ T at 25 °C is more than 5000 s⁻¹ (Millar & Geeves 1983) and this is probably significantly faster than the competing repriming step (AM_o $\cdot$ T $\rightarrow$ AM_c $\cdot$ T). The latter rate has not been measured but the equivalent step in the absence of actin is *ca.* 300 s⁻¹ (Malnasi-Csizmadia *et al.* 2001; Wakelin *et al.* 2002) and the overall rate of the composite hydrolysis step (i.e. open to closed and hydrolysis itself) is slowed by the binding of actin (Rosenfeld & Taylor 1984; White *et al.* 1997). The rate (AM_o $\cdot$ T $\rightarrow$ AM_c $\cdot$ T) is probably no greater than 300 s⁻¹, which would lead to the pathway of dissociation before repriming being greatly favoured.

6. ESTIMATE OF THE WORKING STROKE

The experimentally observed difference in the means of the Gaussian distributions describing the position of the dumbbell when free and when bound to actin, will only correspond to the throw of the cross-bridge if all detected events correspond to the sequence A + M $\cdot$ ADP $\cdot$ P_i $\rightarrow$ AM $\cdot$ ADP $\cdot$ P_i $\rightarrow$ AM + ADP + P_i. There are several other potential sources of events, two of which will be mentioned. The first is that based on solution studies (Sleep & Hutton 1978); we expect *ca.* 10% of interactions to correspond to ATP dissociation and based on the work described above would be associated with no working stroke. In a muscle fibre at saturating [ATP] these would be very brief events of little consequence but at the [ATP] of the trap experiments it is not possible to separate them from product release events. Correspondingly, the throw of the cross-bridge is probably *ca.* 10% greater than the displacement measured in the trap. A second potential source of binding events not associated with a working stroke is the binding to actin of open forms of M $\cdot$ ADP $\cdot$ P_i. We have no idea of the importance of such events but should bear in mind that the observed displacement may well be a significant underestimate. Events of this type are considered in more detail in § 7.

7. CONCLUSIONS AND PROSPECTS

In the simple cross-bridge cycle the myosin product state binds, the lever arm rotates to give the force-generating state and actin is only released after the dissociation of products and the binding of ATP. The reversibility of steps and the possibility of multiple pathways are not generally considered. Intuitively there are two requirements. First, the myosin must bind before undergoing the working stroke. This pathway is favoured by the fact that P_i release from myosin is slow, thus populating M $\cdot$ ADP $\cdot$ P_i, which is predominantly a switch 2-closed, pre-force state. However, we observe that actin binds preferentially to the open form despite M $\cdot$ ATP existing in both open and closed forms in approximately equal concentrations (Wakelin *et al.* 2002). The state M $\cdot$ ADP $\cdot$ P_i is predominantly closed (Wakelin *et al.* 2002), but it has been reported that an ATP analogue gamma-amido-ATP gives an even higher fluorescence, which suggests that the equilibrium can be driven further to the closed form (Wray & Jahn 2002). The idea that there is a non-trivial proportion of the open form of M $\cdot$ ADP $\cdot$ P_i is also consistent with the small equilibrium constants between M $\cdot$ ATP and M $\cdot$ ADP $\cdot$ P_i and between the open and closed states of M $\cdot$ ATP. It would seem that there must be some extra change between M $\cdot$ ATP and M $\cdot$ ADP $\cdot$ P_i, aside from M $\cdot$ ADP $\cdot$ P_i being predominantly closed, which increases the reactivity with actin of the closed form relative to the open form. Second, having undergone the working stroke myosin must not dissociate before product release and dissociation by ATP. This requirement arises because if the cross-bridge dissociates after the working stroke, thermal energy will not be sufficient to allow it to rebind to the same force-producing actin monomer. Dissociation from a post-force state means cessation of tension generation for that cycle of ATP hydrolysis. Consequently, for an efficient muscle the rate of

Figure 7. Rates of actin dissociation from the sequence of intermediates.

dissociation must be significantly less than the rate of turnover of the cross-bridge cycle. For rabbit skeletal muscle the ATPase rate for each myosin head is *ca.* 6 s^{-1} at 20°C (Hilber *et al.* 2001) for the standard initial assumption of all heads gaining equivalent access to actin monomers at the same time. If, as is now becoming increasingly likely, only 20% of the heads can access an appropriate actin (Hopkins *et al.* 1998), the rate would increase to *ca.* 30 s^{-1} .

Knowledge of rates of actin dissociation from the intermediate states is somewhat patchy (see figure 7). The rate from the $\text{AM} \cdot \text{ATP}$ state is well characterized and is *ca.* 5000 s^{-1} (Millar & Geeves 1983), the rate from the other pre-force state, $\text{AM} \cdot \text{ADP} \cdot \text{P}_i$, is not so well characterized but almost certainly also fast (more than 100 s^{-1}). The rates from the two definitive post-force states, AM (*ca.* 0.1 s^{-1}) and $\text{AM} \cdot \text{ADP}$ (*ca.* 2 s^{-1}) are suitably slow. Unfortunately, there is a serious lack of information about the putative force-generating states occurring between $\text{AM} \cdot \text{ADP} \cdot \text{P}_i$ and $\text{AM} \cdot \text{ADP}$. The process of $\text{M} \cdot \text{ADP}$ binding to actin was characterized by Taylor (1991) who found that as expected it consisted of two steps. Actin dissociated rapidly from the intermediate state, $\text{AM}^2 \cdot \text{ADP}$ (see equation (7.1)).

The kinetics have been thoroughly investigated but a superscript 2 has been attached to the state, just to acknowledge the fact that it has not been proven to be on the ATPase pathway. Taylor observed that its general properties were similar to the $\text{AM}' \cdot \text{ADP}$ state, partly characterized by $\text{ATP} \leftrightarrow \text{P}_i$ exchange (Sleep & Hutton 1980). Because the rate of exchange is measured during ATPase activity, it is likely that the $\text{AM}' \cdot \text{ADP}$ state is on the ATPase pathway. $\text{M} \cdot \text{ADP}$ is in the post-force conformation and thus it is very likely that $\text{AM}^2 \cdot \text{ADP}$ state is also post-force, but actin dissociates at a similar speed to the formation of $\text{AM} \cdot \text{ADP}$. If $\text{AM}^2 \cdot \text{ADP}$ were the main force-producing state such rates could result in as much as half the ability to sustain force or carry out work being lost. Important questions remain to be resolved.

While the properties of the post-force $\text{AM}^2 \cdot \text{ADP}$ state are already cause for concern, the situation becomes even more serious if the first force-generating state occurs before P_i release. It can be seen that the rate of dissociation steadily increases as one progresses back through the scheme from AM towards $\text{AM} \cdot \text{ATP}$ (see figure 7). It is worth reviewing the evidence relating to the order of tension generation and P_i release.

- (i) Upon addition of ATP, no burst of free P_i has been observed in either native (modest shortening velocity) or crosslinked (pseudo-isometric) myofibrils. At least under the conditions of the experiments, the fraction of myosin in $\text{M} \cdot \text{ADP}$ or $\text{AM} \cdot \text{ADP}$ states is undetectably

small (Lionne *et al.* 2002). If the concentration of $\text{AM} \cdot \text{ADP}$ states is very low it would imply that the tension generating state must be an $\text{AM} \cdot \text{ADP} \cdot \text{P}_i$ state.

- (ii) Caged P_i experiments were most readily interpreted on the basis of tension generation preceding P_i release (Dantzig *et al.* 1992).
- (iii) The small lag in tension development (5 ms at 20°C) after photolytic release of ATP is not consistent with tension generation occurring after two slow steps (Sleep *et al.* 2004) (hydrolysis *ca.* 100 s^{-1} and P_i release *ca.* 80 s^{-1} (White *et al.* 1997)).
- (iv) The data of Ranatunga *et al.* (2002) describing the effect of $[\text{P}_i]$ on tension transients were significantly better fitted if P_i was released after the force-generating transition (Smith & Sleep 2004). However, those analysing the crystal structure of myosin have tended to favour a mechanism in which P_i is released before force-generation (e.g. Reubold *et al.* 2003).

The rate of actin dissociation from a myosin intermediate is related to the binding constant, which is effectively controlled by the free energy of the actin bound state. The less negative the free energy of the actin bound state, the weaker the likely energy of actin binding and the greater is the likely rate of actin dissociation. If we consider the worst but most likely case of tension generation before P_i release, there are two steps after the first tension generating state, $\text{AM}' \cdot \text{ADP} \cdot \text{P}_i$, before the stable $\text{AM} \cdot \text{ADP}$ state

The first step is P_i release. In general P_i binds much more tightly to myosin than to actomyosin and actin will bind correspondingly less tightly before P_i release. The second step $\text{AM}' \cdot \text{ADP} \rightarrow \text{AM} \cdot \text{ADP}$ has been characterized to only a limited extent but the extra state $\text{AM}' \cdot \text{ADP}$ is required to account for the observation that increasing the concentration of $\text{AM} \cdot \text{ADP}$ during ATPase activity does not increase the rate of $\text{ATP} \leftrightarrow \text{P}_i$ in the manner expected if P_i bound directly to $\text{AM} \cdot \text{ADP}$, implying that it must bind to a higher-energy precursor, $\text{AM}' \cdot \text{ADP}$ (Sleep & Hutton 1980). For $\text{AM}' \cdot \text{ADP}$ to fulfil its proposed role, the equilibrium constant between it and $\text{AM} \cdot \text{ADP}$ must be greater than 10. Both these steps are likely to make the rate of actin dissociation from $\text{AM}' \cdot \text{ADP} \cdot \text{P}_i$ significantly faster than from $\text{AM} \cdot \text{ADP}$ and thus make it likely that the rate is comparable to or faster than the rate of ATP cycling, which would lead to a significant loss of efficiency. The mechanism proposed by (Holmes *et al.* 2003), in which switch 1 opens before switch 2, corresponding to tight binding before the working stroke, is appealing, but for the model to be validated, information about the equilibrium constants and kinetics of actin binding to the post-force states will be required.

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GLOSSARY

- ADP: adenosine 5'-diphosphate
 AM: actomyosin
 AMPPNP: adenosine 5'-(β , γ -imido) triphosphate
 ATP: adenosine 5'-triphosphate
 GTP: guanosine 5'-triphosphate
 ITP: inosine 5'-triphosphate
 M: myosin
 MgATP: the complex of Mg^{2+} with ATP
 NTP: one of the class of nucleoside 5'-triphosphates
 P_i : phosphate
 PP_i : pyrophosphate
 S1: myosin subfragment 1
 T: ATP