Repriming the actomyosin crossbridge cycle

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The central features of the mechanical cycle that drives the contraction of muscle are two translational steps: the working stroke, whereby an attached myosin crossbridge moves relative to the actin filament, and the repriming step, in which the crossbridge returns to its original orientation. Although the mechanism of the first of these is understood in some detail, that of the second has received less attention. Here, we show that repriming occurs after detachment of the crossbridge from the actin, rather than intervening between two actomyosin states with ATP bound [Eisenberg, E. & Greene, L. E. (1980) Annu. Rev. Physiol. 42, 293–309]. To discriminate between these two models we investigated the single-molecule mechanics of the myosin–actin interaction in the presence of ATP analogues such as GTP, for which the hydrolytic step itself limits the actomyosin GTPase rate to a much lower rate than for ATP. The lifetimes of bound states was proportional to 1/[GTP], indicating that during the bound period myosin was in the actomyosin rigor configuration. Moreover, despite the very low actomyosin GTPase, the rate of actin binding and formation of the rigor state was higher than with ATP; it follows that most interactions with actin result in the release of GTP and not of the products, GDP and phosphate. There was no significant movement of the actin during this interaction, so repriming must occur while myosin is dissociated, as in the original Lynn–Taylor scheme [Lynn, R. W. & Taylor, E. W. (1971) Biochemistry 10, 4617–4624].

The mechanical events responsible for muscle contraction are coupled to the actomyosin ATPase cycle. According to the basic scheme of Lynn and Taylor (L-T) (1) (Fig. 1A), in the relaxed state (crossbridge 90° to the filament axes) the myosin (M) is bound to ADP (D) and phosphate (P). The binding of actin (A) to M-D-P₈ leads to the working stroke, accompanied by product release and the formation of the rigor AM₄₅ state. ATP (T) binds to AM to form AM-T, from which actin rapidly dissociates. Hydrolysis then takes place while myosin is dissociated from actin, and the mechanical repriming of the crossbridge, which positions it for the start of the next cycle, is associated with this step. Further research by Eisenberg and collaborators (2) added important details to the scheme. The concentration of Ca²⁺ regulates the binding to actin of the so-called “strong binding” set of myosin states (M and M-D) but has little effect on the binding of the “weak” states (M-T and M-D-P). This finding suggested that these two sets of states bind to actin in different conformations (2), from which it was natural to infer that the transition between these conformations might correspond to the working stroke. Moreover, the free energy change between M-T and M-D-P was very small (3), and the two complexes bound to actin with similar affinities (4), at least 2 orders of magnitude weaker than that of the strong states. The actin association constant appeared to be a marker for the inferred two conformational states. In this case, as argued by Eisenberg and Greene (5), the logical position for the repriming step would be the transition from a strongly bound AM-T₄₅ to a weakly bound AM-T₉₀ state (see Fig. 1B) and not the hydrolysis step as in the original L-T model. This proposal had an attractive symmetry that was further evidenced by the observation that actin is almost as effective in releasing ATP from M-T as in releasing the products from M-D-P (6). At first sight the model appears to suffer from the difficulty that the repriming (effectively a reversal of the working stroke), as well as the forward working stroke, both take place with myosin attached to actin. However, the kinetic asymmetry accounts for this point: the weakly bound, negative-force-generating AM₉₀ state very rapidly dissociates (~5,000 s⁻¹ (7)), whereas the positive-force-generating AM-D state is more long-lived by 2 orders of magnitude (5).

Our purpose here was to determine whether the L-T scheme correctly locates the repriming step, or whether it accords with the alternative proposal of Eisenberg and Greene (5). To distinguish between the two models we needed to establish whether or not repriming takes place while myosin is associated with actin, that is, in the part of the cycle between AM and AM-T. With sufficiently good time resolution this question could be answered by studying interactions with ATP (see Fig. 2a and b) but at present event detection is currently limited to ~10 ms and the direct approach is not viable because the answer is hidden in the gray, temporally unresolved bands (Fig. 2a and b). The difference in positions during the free and bound periods is a measure of the displacement of the AM state with respect to the reference free dumbbell position. For this to be a valid measure of the working stroke, all of the transitions between the dissociated state (M-T ↔ M-D-P) and the rigor state (AM) must be via product dissociation. However, it has been reported (6, 8, 9) that during acto-Sl ATPase ~10% of interactions of the dissociated myosin states (M-T ↔ M-D-P) with actin correspond to ATP release. If this subset of interactions could be identified, they would report on the displacement between the reference

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position (dissociated state) and AM accessed via ATP release and allow assignment of the location of the repriming step. Unfortunately, the amplitude of the brownian noise (SD ~10 nm) relative to that of the working stroke prevents separation, which results in the interactions observed during ATP hydrolysis being a mixture of the product release events of interest (~90%) and the contaminant ATP release events (~10%). This point will be considered in more detail later.

As the ATP release events cannot be separated, is it possible to increase their proportion so as to be dominant? We have investigated the properties of a class of ATP analogues (e.g., GTP, ITP, and ATPγS) known to have favorable kinetic properties. In the case of ATP, the rate-limiting step of the reaction cycle is product release, and the dominant steady-state intermediate is M-D-P. The major action of actin is to bind to this state and accelerate product release, thus circumventing the slow rate-limiting step. However, as noted above, a relatively minor activity of actin is to bind to M-T and accelerate ATP release. The ATP analogues we have investigated share the property that the rate-limiting step of myosin NTPase is the hydrolytic step. The resultant change in the dominant intermediate from M-D-P to M-NTP offers the prospect of the main interaction of actin being to release NTP. This point has been confirmed, both by the trap experiments reported here and independently by biochemical methods.1

The rate of dissociation of actin from AM-ATP is ~5,000 s⁻¹ [extrapolated up to 20°C (5)], and thus the lifetime of AM-NTP

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The ATP analogues, GTP, ITP, and ATP

Results

stroke.

would be of an analogous kind to the conventional working
then the reaction (involving opening of the switch 2 element)

Fig. 4. Interactions in the presence of MgATP. (a) Histogram showing position of bound events during a period in which a myosin was allowed to explore two target zones by moving the dumbbell past the myosin at a uniform velocity. (b) Plot of the SD of bead position against mean bead position for 5 ms time slices of a 100 s period of data collection. Below is an actin filament, scaled to the x axis: the effects of the target zones and the individual actin monomers are clearly evident.

described (15). This method detects binding events, finds the optimum assignments for the rates to and from the low-variance state (conventionally termed f and g), and determines the average variance levels for the two states. From these levels and the covariance of the trapped beads during bound periods, it also calculates the compliances of the myosin and the links between the two trapped beads and the actin filament. The assumption that the behavior can be described by the two rate constants, f and g, is justified by the results (see Figs. 6 and 7). It should be made clear that, in common with almost all previously reported actomyosin single-molecule experiments, we are recording event-averaged working strokes, that is the mean value of the average displacements during each recognized actomyosin interaction. If the displacements during all of the bound events were averaged so as to give the time-averaged working stroke, then, in the absence of an energy source, the answer must be zero. This thermodynamic constraint does not apply to the event-averaged working stroke. The relation of the throw of the crossbridge (commonly termed h) to the observed, event-averaged, working stroke is model dependent, but in the absence of specific information we assume that the relation will be the same for the binding of M-GTP as for M-ADP-PI. This notion is reasonable, as if a displacement did result from M-GTP binding, then the reaction (involving opening of the switch 2 element) would be of an analogous kind to the conventional working stroke.

Results

The ATP analogues, GTP, ITP, and ATPγS, have in general been used to investigate weak interactions (16), but the trap results immediately revealed the presence of strongly bound states (Fig. 5a). The traces are very similar in nature to those observed with ATP. The ratio of the variances of bead position with the actin free and bound was typically >12, indicating that the attached state was mechanically similar to that of the rigor state previously characterized (15, 17). The stiffness of the combined traps was 0.04 pN nm, and the myosin stiffness was typically 50 times greater (≈ 2 pN nm). The bound states of all three ATP analogues studied were found to share this property. As described in Materials and Methods, a characteristic feature of interactions in the presence of ATP is that they can be assigned to specific actin monomers within target zones (12). This behavior was also observed when ATP was replaced by any of the three different substrates, which provides good evidence that interactions are with a single myosin head. Two examples (ATPγS and GTP) are shown in Fig. 5. A kinetic argument can be used to confirm that the low-variance state observed is the rigor state AM for both the analogues and ATP. If the only low-variance state is AM then a plot of the number of events versus event duration will as observed correspond to a single

Fig. 5. Interactions in the presence of MgITP. (a) Four-second record of head position with 100 μM ITP. (b and c) Histograms showing the positions of bound events during a period in which the dumbbell was moved past the myosin at a uniform velocity (b, in the presence of ATPγS; c, in the presence of GTP). The rate of success in achieving this level of resolution was similar (~50%) to that found for ATP. Failure to observe interactions at this resolution was almost certainly caused by either unfavorable attachment of the SI to the surface, high-compliance links between actin and the trapped beads, or occasionally poor operation of the feedback loop used to keep the myosin-coated bead in a constant position with respect to the trapped beads. With good components, interactions could be assigned to individual monomers for essentially all dumbbells tested and most myosins.

Fig. 6. The relative frequency of events plotted against event duration. ATP (○), ATPγS (□), and GTP (●). Concentrations, fitted rates, and total numbers of events were, respectively, 40 μM, 122 s⁻¹, and 4,013; 500 μM, 108 s⁻¹, and 18,963, and 200 μM, 56 s⁻¹, and 13,811. At the relatively high nucleotide concentrations featured in this plot the rates fitted in this manner are somewhat higher than given by the global fit of the hidden Markov method.
exponential. For ATP, the good fit (see Fig. 6) indicates that the lifetime of any low-variance state with bound products is shorter than can be detected by using the variance method of analysis (less than \( \sim 10 \) ms). The plots for GTP and ATP\( \gamma S \) are also well fitted by single exponentials, as expected from the high maximum rate of dissociation at saturating concentrations of these analogues; the occupancy of a low-compliance AM\( \gamma NTP \) state is not evident. The duration of events is thus controlled solely by the rate of nucleotide binding, and Fig. 7 gives a plot of the rates of dissociation as a function of nucleotide concentration. The observed linearity over the experimentally accessible range confirms that AM is the only significant low-variance state. The apparent second-order rate constants for GTP, ITP, and ATP\( \gamma S \), respectively, are comparable to solution values for acto-SI (10, 11). We expect the second-order rate constants deduced from the trap experiments to be very similar to solution values but there is no such clear relation between the second-order rate of actin binding to M-D-P (and M-T) and the rate of occurrence of myosin-binding events, which critically depends on the exact positioning of the dumbbell with respect to the interacting SI molecule. However, the effect of these less controllable parameters will average out over a number of experiments, and for all three alternative substrates, the rate is clearly a few times higher than for ATP (see Table 1).

Most of the ITP experiments were done at a slightly higher ionic concentration and with rapid rebinding events vitiated the operation of the repriming step of the crossbridge cycle by analyzing the partial reaction of the actomyosin cycle between \( A + M-T \rightarrow M+NTP \rightarrow A + T \). For this purpose we used three ATP analogues for which the dominant steady-state intermediate during NTPase activity is M-NTP. The duration of bound events was found to be proportional to \( 1/\text{[ATP analogue]} \) in the experimentally accessible concentration range, as expected if the species giving the stiff, low-noise interaction were the rigor AM state. Actin is a much poorer activator of the myosin

![Fig. 7](image)

Dependence of the rate of dissociation on \([ATP\gamma S], [GTP], \) and \([ITP]\). The GTP and ATP\( \gamma S \) experiments were done with our normal buffer A of 25 mM KCl, 25 mM Hepes, and 4 mM MgCl\(_2\), and the ITP was done in the presence of an additional 25 mM KCl to reduce the rate of binding to actin. The fitted lines have been constrained to a zero rate at zero [nucleotide] although there is evidence of slightly anomalous behavior at very low concentrations.

![Fig. 8](image)

Histograms of the mean positions during each bound period. (a) 50 \( \mu \text{M GTP} \). (b) 100 \( \mu \text{M ATP\( \gamma S \)} \).

**Discussion**

Single-molecule mechanical methods have been used to explore the repriming step of the crossbridge cycle by analyzing the partial reaction of the actomyosin cycle between \( A + M-T \rightarrow M+NTP \rightarrow A + T \). For this purpose we used three ATP analogues for which the dominant steady-state intermediate during NTPase activity is M-NTP. The duration of bound events was found to be proportional to \( 1/\text{[ATP analogue]} \) in the experimentally accessible concentration range, as expected if the species giving the stiff, low-noise interaction were the rigor AM state. Actin is a much poorer activator of the myosin

**Table 1. Working strokes for different myosin nucleotide complexes**

<table>
<thead>
<tr>
<th>Nucleotide, ( \mu \text{M} )</th>
<th>ATP, 10</th>
<th>ITP, 50</th>
<th>ITP</th>
<th>GTP, 200</th>
<th>GTP</th>
<th>ATP( \gamma S ), 200</th>
<th>ATP( \gamma S )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apparent working stroke</td>
<td>5.4</td>
<td>0.5 ± 0.8 nm</td>
<td>0.8 ± 0.6 nm</td>
<td>0.4 ± 0.7 nm</td>
<td>0.6 ± 0.7 nm</td>
<td>0.0 ± 0.2 nm</td>
<td>0.2 ± 0.3 nm</td>
</tr>
<tr>
<td>No. of events</td>
<td>5,580</td>
<td>4,099</td>
<td>27,028</td>
<td>8,399</td>
<td>17,507</td>
<td>9,105</td>
<td>17,252</td>
</tr>
<tr>
<td>No. of records</td>
<td>18 ( \times ) 100s</td>
<td>13 ( \times ) 100s</td>
<td>44 ( \times ) 100s</td>
<td>15 ( \times ) 100s</td>
<td>49 ( \times ) 100s</td>
<td>10 ( \times ) 100s</td>
<td>38 ( \times ) 100s</td>
</tr>
<tr>
<td>Rate ( M^{-1} \mu \text{s}^{-1} )</td>
<td>2</td>
<td>0.16</td>
<td>0.24</td>
<td>0.4</td>
<td>4 ( \times )</td>
<td>4 ( \times )</td>
<td>4 ( \times )</td>
</tr>
<tr>
<td>On rate</td>
<td>1( \times )</td>
<td>5.2 ( \times )</td>
<td>4 ( \times )</td>
<td>4 ( \times )</td>
<td>4 ( \times )</td>
<td>4 ( \times )</td>
<td>4 ( \times )</td>
</tr>
<tr>
<td>No. of myosins</td>
<td>5</td>
<td>3</td>
<td>15</td>
<td>3</td>
<td>12</td>
<td>3</td>
<td>10</td>
</tr>
</tbody>
</table>

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NTPase for these analogues than for ATP. Our observation that the interaction rate is significantly higher with GTP, ITP, and ATP\(S\), than with ATP (see Table 1) provides compelling evidence that almost all interactions are caused by actin binding to M\(\text{NTP}\), leading to dissociation of NTP and the formation of the rigor AM state. It has previously been shown that the addition of actin to an equilibrium mixture of M\(T\) and M\(D-P\) releases ~10% of the bound nucleotide as ATP (6). A similar study showed that in the case of GTP >90% of the bound nucleotide is released as GTP, which confirms our conclusions that the events we observe are associated mainly with GTP release. The contribution of AM-NTP states to the low-noise periods is below the level of detection because the rate of actin dissociation (AM\(\text{NTP} \rightarrow A + \text{M\text{-}NTP}\)) is greater than the time resolution of our method of analysis.

The state AM\(0\) is a postforce 45° state, and we must assume that the first state upon ATP binding is AM\(45\text{-}T\). The reverse path from M\(T\) to AM\(45\text{-}T\) must pass through this state. If the preceding state is M\(T\), no displacement will be observed (L-T), whereas if the preceding state is AM\(0\), a displacement will be observed (Eisenberg-Greene). The relative fluxes from these two sources depend on all of the rates linking the four states: AM\(45\text{-}T\), AM\(0\), M\(45\text{-}T\), and M\(0\). We observe no displacement upon M\(GTP\) binding to actin and forming AM and thus conclude that M-GTP must bind to actin in the same mechanical conformation as the rigor state (45°). Consequently, the repriming step of the contractile cycle (90° to 45° transition) must occur while myosin is dissociated from actin (L-T). The results for ITP and ATP\(S\) were very similar. The observation of a null repriming stroke is only significant if product release is associated with a working stroke but for the analogues, the kinetics are such that almost all of the events correspond to repriming and it is not possible to infer the working stroke associated with product release from trap experiments. In muscle fibers, ITP gives 35% of the tension and 15% of the shortening velocity provided by ATP, which in view of the majority of actin interactions being nonproductive and the turnover rate being slow, is consistent with the working stroke being comparable with that of ATP.

In recent years there have been important developments in relating the structures observed by x-ray crystallography and electron microscopy to the actomyosin ATPase mechanism and the crossbridge cycle. First, the transition from a state with a closed to an open switch 2 element (a feature characteristic of P-loop proteins) was associated with a swing of the lever arm, that is, with the working stroke (90° → 45°; reviewed in ref. 18). Very recently evidence has been presented that the binding of actin to Sl results in a closing of the actin binding cleft and that this is structurally coupled to the opening of switch 1 (again a characteristic element of P-loop proteins) (19–21). The central features of the crossbridge cycle, the working stroke, and the antagonistic behavior of ATP and actin binding to myosin thus finally have a plausible structural interpretation. In retrospect it has become apparent that Eisenberg and Greene’s weak and strong states may well have some relation to states with closed and open switch 1 elements rather than, as they originally conjectured, with the working stroke, that is, with the switch 2 element. The full mechanism may be rather more complicated but this simple scheme accounts for our results and is convenient for the purposes of discussion.

We have previously reported that the binding of the switch 2 open states, M\(\text{PPP}\), M\(\text{ADP}\), and M, to actin, is in no case associated with a detectable working stroke. The only state observed to give a working stroke was the closed state M\(D-P\) (22), and this set of observations fits readily into the proposed structural model, in which a working stroke results exclusively from the opening of switch 2 and not from other events associated with actin binding. An important aspect of the structural model is that the nucleotide pocket must close before the active site is in the appropriate conformation to catalyze hydrolysis. This proposition has been substantiated by fluorescence studies (23, 24), which have given evidence for both open and closed forms of M\(T\). Our results can now be interpreted in terms of the repriming step corresponding to the transition between M\(O\text{-}T\) and M\(c\text{-}T\) (as opposed to AM\(O\text{-}T\) and AM\(c\text{-}T\) for the Eisenberg-Greene proposal).

Is it safe to extrapolate conclusions regarding repriming from these ATP analogues to ATP itself? A potential weakness in the argument arises from the possibility that hydrolysis is rate limiting for the GTP-like analogues because the open to closed transition is much less favorable than for ATP. This process would result in a reduction in observed rate of hydrolysis and populate the M\(O\text{-}T\) state at the expense of M\(c\text{-}T\), thus favoring formation of AM without an observable displacement. Fortunately, it has been reported (25) that the equilibrium between the open and closed forms of M\(\text{NTP}\) for the analogues are all quite comparable to that for ATP (40° ≤ 0.3 ≤ 0.7), and ≤ 1.7 for ATP, ATP\(S\), GTP, and ITP, respectively. A second kinetic argument favors dissociation before repriming for ATP. The rigid state AM is an open state (AMo) and the first ATP state must be AM\(0\). The first-order rate of actin dissociation from M\(c\text{-}T\) at 25°C is >5,000 s\(^{-1}\) (7), which is probably significantly faster than the competing repriming step (AM\(O\text{-}T\) → AM\(c\text{-}T\)). The latter rate has not been measured but the rate of the equivalent step in the absence of actin is ~300 s\(^{-1}\) (23, 25) and the overall rate of the composite hydrolysis step (i.e., open to closed and hydrolysis itself) is slowed by the binding of actin (26, 27). The rate (AM\(O\text{-}T\) → AM\(c\text{-}T\)) is probably more than 300 s\(^{-1}\), which would lead to the pathway of dissociation before repriming being greatly favored.

Physiological experiments (28) continue to indicate that the working stroke is ~10 nm, considerably more than the estimates (~5 nm) from in vitro studies. One small contribution to this discrepancy is that ~10% of the interactions of myosin with actin result in ATP release rather than product release (6) and would be associated with no working stroke. It is likely that the true working stroke of rabbit skeletal myosin is ~10% more than the average displacement observed upon actin binding.

The observation that, of all the myosin intermediate states investigated, M\(D-P\) is the only one to give a working stroke leads to consideration of what constrains myosin to bind in the preforce state before undergoing the working stroke (see Fig. 9). There are two intuitive requirements. The first is that myosin initially binds to actin in a preforce state; the second is that after the working stroke myosin does not dissociate from actin. The second requirement arises because in the postforce conformation thermal fluctuations will not be sufficient to allow repriming.

![Diagram](image)
to the same force-producing actin monomer and another ATP will have to be hydrolyzed before force can be produced again.

Meeting the first requirement is helped by the fact that the rate of Pi release is slow in the myosin ATPase scheme so that the switch 2 closed (preforce) M-D-P is the dominant intermediate. However, the M-T state exists in open and closed forms in equal concentrations and as the equilibrium constant of the hydrolysis step itself is close to 1, it makes it unlikely that the concentration of the open form of M-D-P is negligible. In view of our observation that actin binds to the open form of M-T, even a small percentage of the open form of M-D-P could lead to a serious loss of efficiency as a result of myosin undergoing the working stroke before binding to actin. This finding suggests that some other important change in structure, associated with the hydrolysis step, results in the interaction of actin with the switch 2 closed form of M-D-P being favored.

For the second requirement, the slow rate of actin dissociation from postforce states can be restated in terms of the rate of actin dissociation needing to be less than the overall ATPase rate. Actin is tightly bound to the two states at the end of the cycle, AM-D and AM, and the rates of dissociation are ~1 and 0.1 s⁻¹ (29), comfortably lower than the ATPase rate (~6 s⁻¹). However, the binding constant weakens and the rate of dissociation increases as one progresses back through the cycle to M-D-P (probably ~1,000 s⁻¹). Taylor (29) characterized the initial AM-D state formed upon M-D binding to actin and the rate of actin dissociation was >150 s⁻¹. It has not been possible to prove that this state is on the ATPase pathway but its properties are rather similar to the AM-D state partially characterized by measurement of ATP ↔ Pi exchange during the hydrolysis of ATP (30).

A slow rate of actin dissociation goes along with tight actin binding and the simplest scheme would be one in which actin was bound tightly to a closed myosin state before the working stroke. This finding is in accord with the model deduced from recent crystallographic and electron microscopic observations (19–21) in which switch 1 opens, promoting tighter actin binding before the working stroke (switch 2 opening). However, a considerable fraction of the energy of ATP hydrolysis is associated with Pi release (31), which means that actin binding does not really become tight until after Pi release. It is thus surprising that both physiological and biochemical evidence favors a working stroke before the release of Pi (32, 33).