Phosphate enhances myosin-powered actin filament velocity under acidic conditions in a motility assay

Edward P. Debold,1 Matthew A. Turner,1 Jordan C. Stout,1 and Sam Walcott2

1Department of Kinesiology, University of Massachusetts, Amherst, Massachusetts; and 2Department of Mechanical Engineering, Johns Hopkins University, Baltimore, Maryland

Submitted 24 November 2010; accepted in final form 22 February 2011

Debold EP, Turner MA, Stout JC, Walcott S. Phosphate enhances myosin-powered actin filament velocity under acidic conditions in a motility assay. Am J Physiol Regul Integr Comp Physiol 300: R1401–R1408, 2011. First published February 23, 2011; doi:10.1152/ajpregu.00772.2010.— Elevated levels of inorganic phosphate (P_i) are believed to inhibit muscular force by reversing myosin’s force-generating step. These same levels of Pi can also affect muscle velocity, but the molecular basis underlying these effects remains unclear. We directly examined the effect of Pi (30 mM) on skeletal muscle myosin’s ability to translocate actin (V_{actin}) in an in vitro motility assay. Manipulation of the pH enabled us to probe rebinding of Pi to myosin’s ADP-bound state, while changing the ATP concentration probed rebinding to the rigor state. Surprisingly, the addition of Pi significantly increased V_{actin} at both pH 6.8 and 6.5, causing a doubling of V_{actin} at pH 6.5. To probe the mechanisms underlying this increase in speed, we repeated these experiments while varying the ATP concentration. At pH 7.4, the effects of Pi were highly ATP dependent, with Pi slowing V_{actin} at low ATP (<500 μM), but with a minor increase at 2 mM ATP. The Pi-induced slowing of V_{actin} evident at low ATP (pH 7.4), was minimized at pH 6.8 and completely reversed at pH 6.5. These data were accurately fit with a simple detachment-limited kinetic model of motility that incorporated a Pi-induced prolongation of the rigor state, which accounted for the slowing of V_{actin} at low ATP, and a Pi-induced detachment from a strongly bound post-power-stroke state, which accounted for the increase in V_{actin} at high ATP. These findings suggest that Pi differentially affects myosin function: enhancing velocity, if it rebinds to the ADP-bound state, while slowing velocity, if it binds to the rigor state.

MUSCULAR FORCE AND MOTION are generated through the cyclical interaction of actin and myosin, in a process ultimately powered by the hydrolysis of ATP (Fig. 1). In the 1980s, several investigations established that elevated levels of organic phosphate (P_i) reduced muscular force, leading to the notion that P_i release by myosin was closely associated with force generation (12) and the rotation of the lever arm (2). This hypothesis postulates that P_i rebinds to myosin (M) in a state in which myosin is strongly bound to both actin (A) and ADP (AM,ADP) and, in one or more steps, reverses the force-generating step, leading to an increase in the population of the weakly bound myosin, in the M.ADP,P_i state (32). While competing theories exist (3), models based on the P_i-induced detachment can account for much of the effect on muscular force (26). However, the effects of P_i on muscle’s shortening velocity have been more difficult to explain using the same model.

While the earliest experiments in muscle fibers suggested that Pi had no effect on unloaded shortening velocity (V_{us}) (7), subsequent efforts revealed that P_i could induce either a minor increase in velocity, if the ATP level were high, or a profound decrease in velocity at micromolar levels of ATP (27). The molecular basis of these unexpected findings was probed by employing the in vitro motility assay, where isolated myosin bound to a coverslip translocates fluorescently labeled actin filaments, an assay considered analogous to V_{us} in muscle fibers. In agreement with the findings in muscle fibers (27), P_i was found to cause a mild increase in actin filament velocity (V_{actin}) at saturating ATP (14). Also in agreement with observations in muscle fibers, several authors reported that elevated levels of Pi decrease V_{actin} at micromolar levels of ATP in the in vitro motility assay (1, 15, 37).

Since the P_i-induced increases in velocity have typically been small and not consistently observed, researchers have focused on identifying the molecular basis of the depressive effects of P_i on V_{actin}. Pate and Cooke (27) were the first to suggest that this effect was explained by P_i competing with ATP for the active site of myosin. Assuming that V_{actin} is proportional to myosin’s unitary displacement (d), divided by the duration of strong actin binding (t_{on}), P_i binding to the active site could prevent ATP-induced detachment, prolonging t_{on} and thereby slowing V_{actin} (37). While this explanation was consistent with much of the data, it remains controversial, evidenced by a recent challenge that proposed a distinctly different underlying molecular basis of this phenomenon (15).

Hooft et al. (15) proposed that the P_i-induced decrease in V_{actin} at subsaturating ATP is most accurately explained using a thermodynamic approach, where V_{actin} is governed, not solely by detachment kinetics, but also is significantly influenced by the amount of free energy (ΔG) available to myosin to drive filament motion (e.g., ΔG = −RT ln [P_i][ADP]/[ATP]), where R is the gas constant, T is the absolute temperature, and brackets denote concentration. Raising P_i, they postulated, reduces ΔG, decreasing myosin’s ability to drive filament motion, and thus causes V_{actin} to slow. The depressive effect on V_{actin} would only be evident below a critical concentration of ATP (and thus ΔG), providing a rationale for the lack of an effect at millimoles of ATP. However, the underlying assumptions of this model and the conclusions reached were subsequently challenged (1).

Amrute-Nayak et al. (1) incorporated fluorescently labeled ATP to track the lifetime of a nucleotide in myosin’s catalytic site in a single molecule total internal reflectance fluorescence microscopy assay (i.e., TIRF). They found that P_i did not
significantly prolong the lifetime of the nucleotide in the active site, suggesting that Pi does not rebind to the AM.ADP state, but rather rebinds to the rigor state (AM), creating an AM.Pi state. This offered renewed support, a detachment-limited explanation of the effects of Pi, reaffirming the notion that Pi slows V$_{actin}$ by prolonging t$_{on}$.

The equivocal findings at high ATP and the contrasting explanations for the Pi-induced depression in V$_{actin}$ demonstrate that the mechanisms that underlie Pi rebinding to myosin remain unclear. Given the close association between Pi and the PI-induced decrease in myosin function in an in vitro motility assay (8). This finding provides a unique opportunity to determine whether Pi can rebind to the ADP state under these conditions and what effects it might have on myosin function. Therefore, by systematically altering the pH, [ATP], and [Pi], conditions and what effects it might have on myosin function.

![Fig. 1. A schematic model of myosin's cross-bridge cycle that links the biochemical and mechanic events. The $d$ refers to myosin's unitary displacement, and $t_{on}$ refers to the duration of strong actomyosin binding. $t_{ADP}$ and $t_{rigor}$ refer to the duration of the actin (A) myosin (M), ADP and AM states, respectively (24). Putative states of Pi rebinding are indicated with arrows.](image-url)

methods

Proteins

All animal tissue was obtained in accordance with University and NIH policies and using a protocol submitted to and approved by the institutional animal care and use committee at the University of Massachusetts.

Skeletal muscle myosin was isolated from chicken pectoralis tissue based on a method previously described (21). SDS-PAGE gels (data not shown) demonstrated clear bands for myosin heavy chain and both the regulatory and essential light chains, with faint bands elsewhere in the gel, confirming the high purity of the isolation. Prior research demonstrates that myosin from this muscle of chicken expresses, almost exclusively, a fast isoform of myosin heavy chain (4).

Following purification, the myosin was diluted in 50% glycerol (vol/vol) and stored at $-20^\circ$C. An additional purification was done on the day of the experiment by adding an equimolar amount of filamentous actin and 2 mM ATP and then centrifuging the sample at 400,000 g for 20 min. In this step, inactive, rigor-like myosin heads pellet with the filamentous actin, and the supernatant was used for the motility assay.

Actin was purified from chicken pectoralis muscle, based on the methods described (25), with SDS-PAGE gels used to quantify the purity of each isolation. To stabilize the actin in filamentous form and visualize it in epi-fluorescence, phalloidin with tetramethylrhodamine isothiocyanate (Sigma-Aldrich, St. Louis, MO) was added to 1 μM actin and incubated overnight at 4°C, as previously described (36).

buffers

On the day of an experiment, the myosin was diluted from its highly concentrated level (~30 mg/ml) in 50% glycerol to 200 μg/ml in myosin buffer (MB; composed of 300 mM KCl, 25 mM imidazole, 1 mM EGTA, 4 mM MgCl$_2$, 1 mM DTT). Due to its charge, manipulation of the [Pi] necessitated controlling the ionic strength of the motility solutions, while varying the ATP and pH. This was done by adjusting the [KCl] in each buffer to maintain a constant total ionic strength of 125 mM. The constituents of all motility buffers were calculated using WinMax Chelator with the stability constants provided within the program (29). Methylcellulose (1%) and an oxygen scavenging system (1,725.5 mg glucose, 7.5 mg glucose oxidase, and 1.35 mg catalase, in 600 μl double-distilled H$_2$O) were also added to the final motility buffer to keep the actin filaments in contact with the surface and prevent rapid photo-bleaching, respectively.

We assume that, in the 0 mM Pi condition, the [Pi] was actually ~0.5 mM due to contamination and hydrolysis (7). In addition, we also assumed 0.025% contaminating pyrophosphate (inorganic pyrophosphate), which has a higher affinity for myosin than Pi (1). However, recent evidence indicates that this amount of inorganic pyrophosphate contamination has no discernable effect on myosin function in an in vitro motility assay (1).

in vitro motility assay

In the in vitro motility assay was performed as previously described (18), with minor modifications. Briefly, myosin was introduced into a nitrocellulose-coated flow cell in MB and allowed to bind for 1 min and then treated with 0.5 mg/ml bovine serum albumin (in AB) to block any uncoated areas of the surface. The flow cell was then washed with AB, and an actin coat (1 μM unlabeled actin in AB) was applied to prevent any remaining rigor-like myosin molecules from reattaching $V_{actin}$. This was followed by a wash with 1 mM ATP in AB.
to release any actin bound to active myosin molecules. The flow cell was then washed with AB without ATP, and then tetramethylrhodamine isothiocyanate-labeled actin filaments (~5 nM in AB) were applied and allowed to bind to the myosin for 1 min. Any actin not bound to myosin was removed with another AB wash. The final actin was manually tracked using the MTrackJ plug-in for ImageJ (National Institutes of Health Image). Typically, the paths of eight individual filaments were tracked for each 30-s time block (or more if the frame rate was reduced to 5–10 frames/s to increase the distance over which a filament was tracked).

The temperature of the flow cell was maintained at 30°C using an objective temperature controller (20/20 Technologies, Wilmington, NC). Fluorescent actin filament motion was visualized using an intensified charge-coupled device camera (model XR/MEGA-10EX S30, Stanford Photonics, Palo Alto, CA) mounted to a Nikon Eclipse Ti inverted microscope with a ×100, 1.4 NA CFI Plan Apo objective. Video was captured by coupling the intensified charge-coupled device camera to an Epix-LVDS frame grabber (Epix, Buffalo Grove, IL). The camera and frame grabber were controlled by a personal computer using PIPER Control software (Stanford Photonics). Three 30-s time blocks of data were obtained at 30 frames/s for each condition, with the exception of conditions eliciting very low velocities (e.g., low ATP and low pH). Under conditions that elicited slower velocities, the frame rate was reduced to 5–10 frames/s to increase the distance over which a filament was tracked.

Analyses

$V_{\text{actin}}$ was manually tracked using the MTrackJ plug-in for ImageJ (National Institutes of Health Image). Typically, the paths of eight individual filaments were tracked for each 30-s time block (or more if the frame rate was reduced) with 25–35 points typically needed to accurately trace the path of a filament over the entire recording. The average velocity of 50–80 individual filaments, for each condition, was compiled and then used for statistical comparisons.

All filaments under each level of $pH$ and $P_i$, at 2 mM ATP, were analyzed using a two-way ANOVA (pH × $P_i$), with the $\alpha$-level set at $P < 0.05$ in SigmaStat (Systat Software, San Jose, CA). The ATP curves were fit with a Michaelis-Menten relationship where:

$$V_{\text{actin}} = \frac{V_{\max}[\text{ATP}]}{K_M + [\text{ATP}]}$$

where $V_{\max}$ is the maximal $V_{\text{actin}}$ at infinite [ATP], and $K_M$ is the [ATP] at which $V_{\text{actin}}$ is one-half $V_{\max}$. The analysis was performed using SigmaPlot 8.0 (Systat Software, San Jose, CA), employing a least squares fit.

RESULTS

As expected, at 2 mM ATP, $V_{\text{actin}}$ progressively decreased with increasing levels of acidosis (Fig. 2), with $V_{\text{actin}}$ decreasing by 44% when the $pH$ was lowered from 7.4 to 6.8 and decreased by a further 30% when the $pH$ was lowered to 6.5. However, elevating $P_i$ to 30 mM under acidic conditions recovered much of this loss in $V_{\text{actin}}$, with $V_{\text{actin}}$ increasing by 26% at $pH$ 6.8 and more than doubling at $pH$ 6.5. There was even a small but significant increase in $V_{\text{actin}}$ at $pH$ 7.4 with the addition of 30 mM $P_i$, confirming a strong $pH$ dependence of this effect. The $pH$ dependence of the effect of $P_i$ was quantified using a two-way ANOVA and revealed a significant $pH \times P_i$ interaction, with the effects at $pH$ 6.5 being significantly ($P < 0.05$) greater than those at both 6.8 and 7.4.

ATP Curves

To investigate the kinetic basis of these observations $V_{\text{actin}}$ was measured as a function of [ATP], $pH$, and $[P_i]$ (Fig. 3). We started by quantifying the effects of increasing acidosis alone, and, as expected, acidosis depressed $V_{\text{actin}}$ at any given [ATP]. This resulted in a decrease in $V_{\max}$ at both $pH$ 6.8 and 6.5 compared with $pH$ 7.4. $K_M$ was also affected by acidosis with $pH$ 6.8, causing a mild decrease. The effect on $K_M$ at $pH$ 6.5 was difficult to quantify because the no-filament motion was detected <500 μM ATP. Due to this issue, the curve is poorly

![Fig. 3. $V_{\text{actin}}$ vs. ATP. Data points are means ± 95% confidence interval. Data points without added $P_i$ are solid circles, and corresponding line fits are solid lines. Data points for 30 mM added $P_i$ are shaded squares, and corresponding fits are dashed shaded lines. Points were fit to a Michaelis-Menten relationship using a least squares fitting algorithm (SigmaPlot 8.0). At $pH$ 7.4, the mean ± SE of the fit parameters were 7.1 ± 0.2 and 8.2 ± 0.3 μm/s for maximal $V_{\text{actin}}$ ($V_{\max}$), and 372 ± 40 and 773 ± 86 μM for $K_M$ at 0 and 30 mM $P_i$, respectively. At $pH$ 6.8, $V_{\max}$ was 3.2 ± 0.1 and 5.6 ± 0.2 μm/s, and $K_M$ 119 ± 24 and 687 ± 55 μM at 0 and 30 mM $P_i$, respectively. At $pH$ 6.5, $V_{\max}$ was 2.2 ± 0.1 and 3.9 ± 0.1 μm/s, and $K_M$ 821 ± 192 and 202 ± 28 μM at 0 and 30 mM $P_i$, respectively.](image-url)
simple kinetic analysis to determine the effect of Pi on both the ATP, were small (less robust than the much more pronounced increases in effect is near the typical error for motility measures, making it

conditions of 653 s

constructed a double reciprocal plot to derive the rate constants (rigor time) (24). Assuming a value for

duration of strong actin binding can be further dissected into

rates (Table 1). Inherent in this analysis is the idea that

ADP release (i.e., V

ADP) and ATP-induced dissociation

unaffected by either high levels of Pi (2) or by low pH (8) at

threefold, decrease at pH 6.5 (Table 1). In contrast, added Pi increased the ADP release rate at each pH, with the effects being significant (P < 0.05) at both pH 6.8 and 6.5. In fact, this rate doubled, with the addition of Pi, at 6.8 and tripled at pH 6.5.

k

ATP demonstrated a differential response when pH alone was manipulated, increasing slightly at pH 6.8 compared with pH 7.4, but slowing at pH 6.5. In contrast, elevated Pi caused k

ATP to decrease at each level of pH. This result suggests that elevated levels of [Pi] significantly depress the rate of k

ATP of myosin. This is consistent with the idea that Pi readily competes with ATP for binding to myosin’s active site (1, 27).

DISCUSSION

We have used the in vitro motility assay to probe the molecular basis of the effects of Pi on the actomyosin interaction. We found that the effects of Pi on V

actin are dependent on both ATP and pH, with elevated Pi depressing V

actin at low ATP and high pH, while, at high ATP, phosphate reduces the depressive effects of low pH. While the former effect was expected based on previous findings (37), the later was an unexpected and novel observation.

Michaelis-Menten fits to our data at pH 7.4 indicate that Pi increases the K

m, due to the Pi-induced decrease in ATP to decrease at each level of pH. This result suggests that ADP release rate constant; k

ATP, ATP-induced dissociation rate constant. *Significant (P < 0.05) effect of 30 mM Pi. †Significant effect of pH alone.

Table 1. Derived rate constants for k

ADP and k

ATP

| pH | [Pi], mM | k

ADP (95% CI) \( \times 10^6 \) M

s

| k

ATP (95% CI) \( \times 10^6 \) M

s

| K

d | K

m |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>0</td>
<td>653 (598–718)</td>
<td>1.85 (1.78–1.92)</td>
</tr>
<tr>
<td>30</td>
<td>830 (814–866)</td>
<td>0.99 (0.96–1.03)</td>
<td>6.5</td>
</tr>
<tr>
<td>6.8</td>
<td>0</td>
<td>132 (143–199)†</td>
<td>0.36 (0.26–0.56)†</td>
</tr>
</tbody>
</table>

Values are means with 95% confidence intervals (CI) in parentheses. [Pi], inorganic phosphate concentration; k

ADP, ADP release rate constant; k

ATP, ATP-induced dissociation rate constant. *Significant (P < 0.05) effect of 30 mM Pi. †Significant effect of pH alone.

Kinetic Analysis

Double reciprocal plots of the V

actin vs. ATP data enabled a simple kinetic analysis to determine the effect of Pi on both the ADP release (k

ADP) and ATP-induced dissociation (k

ATP) rates (Table 1). Inherent in this analysis is the idea that V

actin is a detachment-limited process (16), where V

actin is proportional to myosin’s unitary step size (d) divided by the duration of actin strong binding (t

on), i.e., V

actin = dt

on. In this model, the duration of strong actin binding can be further dissected into the time required for ADP release by myosin, plus the time required for ATP to bind and cause dissociation from actin (rigor time) (24). Assuming a value for d of 10 nm (10), we constructed a double reciprocal plot to derive the rate constants for ADP release (k

ADP) and the second-order k

ATP constants under each condition (Fig. 4). Plotted on the y-axis is d/V

actin or t

on, and 1/[ATP] on the x-axis. A linear fit to the data provides a y-intercept (equal to 1/k

ADP) and a slope (equal to 1/k

ATP)(2). Our derived value for k

ADP under control conditions of 653 s

is similar to previous estimates from single-muscle fibers (38) and in vitro motility experiments (15). In addition, the k

ATP of 1.85 \( \times 10^6 \) M/s, which, while slightly lower than previous values derived in the motility assay (2, 15), is similar to values measured in solution (22).

These results show that the rate of ADP release by myosin is decreased in a pH-dependent manner, with the rate decreasing by one-half from pH 7.4 to 6.8 and a further, almost

constrained in this region, and, therefore, estimates of K

m should be interpreted cautiously.

As expected, elevating Pi depressed V

actin at subsaturating ATP, consistent with previous observations (1, 15, 37). This effect, which was strongest at pH 7.4, was still present, albeit smaller in magnitude, at pH 6.8 and was completely reversed at pH 6.5 (Fig. 3). This Pi-induced enhancement of V

actin at pH 6.5 was particularly striking at 300 \( \mu \) M ATP, where myosin was completely incapable of moving actin in the absence of Pi, but moved the actin filaments at near the maximum value under these conditions (3.2 \( \mu \) m/s) in the presence of high Pi.

The increases at pH 7.4, although significant at 2 and 4 mM ATP, were small (~8–10%) and not consistently observed at all ATP levels, for example, 5 mM ATP. This magnitude of an effect is near the typical error for motility measures, making it less robust than the much more pronounced increases in V

max observed at pH 6.8 and 6.5. Elevating phosphate increased K

m values at both pH 7.4 and 6.8, suggesting a lower affinity for ATP. However, the uniform increase in V

actin caused by Pi at pH 6.5 resulted in a decreased K

m.

Fig. 4. Double reciprocal of ATP curves. Data points without added Pi, are solid circles, and corresponding line fits are solid lines. Data points for 30 mM added Pi, are shaded squares, and corresponding fits are dashed shaded lines. V

actin was divided by a d value of 10 nm (10) to estimate t

on. This was plotted against 1/ATP and fit to best fit using the equation t

on = 1/k

ADP + 1/k

ATP [ATP], where brackets denote concentration. Values represent mean ± 95% CI. The estimates for the ADP release rate (k

ADP) and ATP-induced dissociation (k

ATP) under each condition are displayed in Table 1.
high levels of ATP. This, therefore, suggests that the Pi-induced slowing of $V_{\text{actin}}$ is attributable to a prolongation of $t_{\text{on}}$. Since $t_{\text{on}}$ is believed to be composed of the AM.ADP and the rigor (AM) lifetimes, this change could more specifically be attributed to a prolongation of either biochemical state. At low levels of ATP (µM levels), the duration of the rigor lifetime dominates $t_{\text{on}}$ (2); therefore, the depressive effects of Pi on $V_{\text{actin}}$ observed at low ATP are likely due to an effect of Pi on the rigor state. A simple explanation for this observation is that Pi rebinds to actomyosin in the rigor state, prolonging its lifetime through the formation of an AM.Pi state. This would lead to an increase in $t_{\text{on}}$ and thus cause $V_{\text{actin}}$ to slow in a detachment-limited model of $V_{\text{actin}}$.

We explored the molecular basis of this Pi-induced slowing of $V_{\text{actin}}$ further by modifying a simple detachment-limited model of motility, incorporating modifications to myosin’s mechanochemical cycle (see supplemental material; the online version of this article contains supplemental data). We then used this model to simulate fits to the $V_{\text{actin}}$ vs. ATP (Fig. 5). These simulations revealed that the inclusion of an AM.Pi state was required to reproduce the Pi dependence of $K_m$ observed at pH 7.4 and 6.8. We used this same model to probe the molecular basis of the Pi-induced increase in $V_{\text{actin}}$; however, the addition of an AM.Pi state to the simple detachment-limited model could not capture this aspect of the data (Fig. 5A). This was especially evident at low pH (Fig. 5A). Indeed, capturing this aspect of the data required the inclusion of a Pi-induced detachment from a post-power stroke, AM.ADP state (Fig. 5B). However, attempts to use this version of the model on the full data set failed to reproduce the depressed $V_{\text{actin}}$ at low ATP (Fig. 5B). Therefore, the inclusion of both modifications of the model was required to fully reproduce both the depressive effect of Pi at low ATP and the enhancement of $V_{\text{actin}}$ at high ATP (Fig. 5C). This suggests a novel view of how Pi rebinding affects myosin’s mechanochemical cycle to produce distinctly different effects on its function. While more complex models could likely be constructed to capture these differential effects of Pi, this modified detachment-limited model provides a simple mechanistic explanation for our data.

Relevance to Previous Work

Our observation that Pi decreases $V_{\text{actin}}$ at low ATP, increasing the $K_m$ for ATP, is consistent with previous reports in both the motility assay (1, 15, 37) and in single fibers (27). Likewise, our suggestion that Pi and ATP compete for binding to myosin’s nucleotide binding site was originally proposed by Cooke and Pate (27), based on work in muscle fibers, and has subsequently been invoked by others to account for similar observations in the analogous in vitro motility assay (1, 37). Thus our data and model simulations provide further evidence to suggest that Pi can readily rebind to myosin’s empty nucleotide binding pocket. However, our data also suggest that Pi can, under very different conditions, rebind to the myosin in an AM.ADP state.

Pi can rebind to myosin in the AM.ADP state. Our own previous work, using a single-molecule laser trap assay, indicates that acidosis prolongs $t_{\text{on}}$ by prolonging the lifetime of the AM.ADP state, with little effect on the $k_{\text{ATP}}$ rate (8). It is also well established that the rigor lifetime of myosin is ATP dependent (2). This, therefore, strongly suggests that, under our conditions of low pH and high ATP, $t_{\text{on}}$ is dominated by the AM.ADP lifetime; thus it is this state that Pi likely rebinds to under these conditions.

This conclusion directly contradicts the suggestion by Amrute-Nayak et al. (1) that the rebinding of Pi to the AM.ADP state is highly unfavorable under the unloaded conditions of a motility assay. However, our contradictory conclusions may simply be the result of the differing conditions between the two assays. Amrute-Nayak et al. examined the effects of Pi rebinding to myosin at pH 7.0 and nanomolar levels of ATP, conditions that strongly favor rebinding to the rigor state over the AM.ADP state. Also, as noted above, we see exactly the same depressive effect of Pi under similar conditions in the present study (Fig. 3A). However, at low pH and high ATP, where $t_{\text{on}}$ is likely dominated by the AM.ADP lifetime, we observe the opposite effect on $V_{\text{actin}}$, suggesting that Pi can readily rebind to the AM.ADP state, even under unloaded

---

**Fig. 5.** In silico fits to experimental data. Fits to the data are from computer simulations using a mechanistic kinetic model (see supplemental material). The Pi data points are shaded squares, and corresponding fits are the dashed shaded lines, whereas the data points in the absence of Pi are solid circles, and corresponding line fits are solid. The fits in A include an AM.Pi state, but no rebinding to, and dissociation from, a post-power stroke AM.ADP state. B: our fits to the data without an AM.Pi state, but include Pi-induced dissociation from AM.ADP in a post-power stroke state. The fits in C include the addition of both an AM.Pi state and a Pi-induced post-power stroke dissociation from AM.ADP state.
of ADP release, suggested by this kinetic analysis, is more composed of the ADP and rigor lifetimes. Thus the increased rate of previous observations of both pH 7.4 is both qualitatively and quantitatively consistent with raising Pi and lowering pH, in the present study, we presum-
ing a pronounced increase in Pi under acidic conditions. They, however, did not attribute the effects to Pi, because they manipulated other constituents of the assay in addition to Pi, most notably ADP. These differences in assay constituents led them to attribute the increase in V_{actin} to a complex interaction between the added ADP and the effects of acidosis on the rigor state. Since we did not manipulate ADP, we can attribute the findings directly to the effects of Pi.

This increase in V_{actin} in response to Pi is also difficult to reconcile with the mechanism proposed by Hooft et al. (15), who suggested that increasing Pi lowers V_{actin} by affecting the ΔG available to drive actin filament motion. However, by raising Pi and lowering pH, in the present study, we presumably decreased ΔG, but observed an increase in V_{actin} rather than a decrease, as their model might predict. This deviation of our results from their model might indicate that the energetics governing V_{actin} are more complex in our assay due to the additional manipulation of pH.

One potential mechanism to explain the increased V_{actin} comes from the double reciprocal plots of our V_{actin} vs. ATP data (Fig. 4), which suggests that Pi accelerates the rate of ADP release from myosin (Fig. 4, Table 1). However, the presence of additional states and transitions, necessary in our model simulations, means that the assumptions of this kinetic analysis may be invalidated. For example, if Pi rebinding causes dissociation of myosin from actin before it progresses through ADP release and rigor, it would negate the notion that on is composed of the ADP and rigor lifetimes. Thus the increased rate of ADP release, suggested by this kinetic analysis, is more likely the result of a Pi-induced detachment from a strongly bound actin state, rather than an actual speeding of the ADP release rate.

In fact, a Pi-induced detachment from the AM.ADP state is most similar to the mechanism originally proposed by Hibberd et al. (12) to explain the depressive effects of Pi on force in muscle fibers. However, their mechanism implies that Pi re-binding also reverses the rotation of the lever (32), a fact that is difficult to reconcile with the Pi-induced increase in V_{actin}. Reversal of the lever arm rotation would also reverse the previous displacement of the actin filament. In a simple detachment-limited model where V_{actin} = dt_{on}, this predicts that elevated Pi would decrease rather than speed V_{actin}. This, therefore, leads us to suggest that Pi rebinding does not necessarily reverse myosin’s power stroke, but rather induces detachment from actin in a post-power stroke state. In this case, since the displacement of the actin filament from myosin’s power stroke is maintained while t_{on} is decreased, V_{actin} increases.

This proposed mechanism creates a situation in which the release of Pi and ADP would occur from a weakly attached state, a process normally thought to proceed very slowly (20). It would also require that myosin exist in weakly attached states that are thought to possess high affinities for actin (e.g., M.ADP). While there is little structural evidence for a low-affinity M.ADP state (13), indirect evidence exists suggesting that the release of Pi and ADP can indeed occur quite rapidly off actin under certain conditions. For example, elevated levels of Pi have a much greater effect on force than the ATPase rate in muscle fibers (30, 31), suggesting product release continues to be quite rapid off actin. In fact, efforts to model these data appear to require the relatively rapid release of Pi and ADP from myosin while off actin (19) to accurately fit the ATPase results. For a structural viewpoint, it was suggested that, since...
Pi-induced detachment occurs following a weak-to-strong transition, the active site remains in a configuration that favors the rapid release of both Pi and ADP.

Pi reduces myosin’s duty ratio at high ATP. One prediction of the proposed mechanism that Pi can rebind to the AM.ADP state and cause dissociation from strongly bound state, while still being able to complete the ATPase cycle, is that the process would decrease myosin’s duty ratio (the proportion of the ATPase cycle spent strongly bound to actin). This assumes, of course, that the total cycle time is either unchanged or increased, which seems robust based on the effects of Pi of myosin’s ATPase rate in fibers (30, 31). Therefore, to test this prediction of our model, we measured the effect of Pi on myosin’s duty ratio at 2 mM ATP. This was done using the method of Uyeda et al. (33), in which $V_{actin}$ is determined at limiting amounts of myosin. As predicted by the model, elevated Pi reduced the estimate of myosin’s duty ratio. At pH 7.4, Pi reduced the duty ratio from 3.5 to 2.8% (Fig. 6). The Pi-induced reduction in the duty ratio was even more pronounced at pH 6.5, where it was decreased to 2.0%. These experimental observations add further support to the notion that Pi can induce detachment from the post-power stroke AM.ADP state.

Conclusions

In the in vitro motility assay, we have demonstrated that elevated levels of Pi can have distinctly different effects on $V_{actin}$, depending on ATP and pH, slowing $V_{actin}$ at low ATP and high pH, while having the opposite effect at high ATP and low pH. These findings suggest that Pi can rebind to myosin in either the AM.ADP or rigor states. The differential binding provides insight into the molecular basis of the effects on actomyosin function. Specifically, slowing of $V_{actin}$ at low ATP can be explained in a model in which Pi competes with ATP to form an AMPi state. In contrast, the enhancement of $V_{actin}$ can be explained by a model in which Pi induces detachment from a post-power stroke AM.ADP state.

The structural correlates of these Pi-bound states are unclear; however, the known structural differences between the AM.ADP and AM states (13) may impact how Pi rebinds to myosin’s active site and what effects it might have on its function. For example, the release of ADP from the AM.ADP state is thought to be facilitated by a closed-to-open transition in myosin’s active site, and these rearrangements are thought to be communicated through the converter region to the lever arm (34). These subtle changes in the active site might also modulate the nature Pi-rebinding to the active site and impact the functional effects on actomyosin motility. Related to this idea, recent molecular dynamic simulations suggest that there may be multiple pathways of Pi release from the active site (5). This leads to the speculation that the rebinding of Pi might also have multiple routes back to the active site and possibly exert different functional effects on myosin based on pathway accessed.

Perspectives and Significance

Our results suggesting that Pi can rebind to myosin in more than one biochemical state might indicate that the rebinding of Pi to myosin’s active site is more promiscuous than previously believed. Thus the internal atomic motions within the active site that govern Pi rebinding may be much more dynamic than the static high-resolution structures might imply. This notion is supported by recent observations that opening and closing of myosin’s actin-binding cleft is highly dynamic in solution (17). Future work should attempt to capture the dynamic nature of Pi release and rebinding within myosin’s active site to provide a more detailed picture of how Pi is coupled to the generation of force and motion during contraction. Since our conditions of high Pi and low pH are similar to those experienced during intense contractile activity (6), the results also have potential implications for the molecular basis of muscular fatigue. During fatigue, elevated levels of Pi and H\(^+\) are thought to act additively to inhibit contraction, largely based on the effects on maximal isometric force (6). Since force is related to the number of attached cross bridges, our suggestion of a Pi-induced detachment implies that the increase in velocity may come at the expense of force; thus our finding remains consistent with the notion that these ions can additively inhibit muscular force.

ACKNOWLEDGMENTS

We thank Arumina Guha and Jennifer Harkey for helping with protein preparation, data collection, and analysis. We also thank Dr. Stephanie L. Jones for assisting in the editing of the manuscript.

GRANTS

This work was supported by funding from the University of Massachusetts, Amherst (PFRG0000000062) and the American Heart Association (09SDG2100039), both awarded to E. P. Debold.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES