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

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Running head: Effects of phosphate and acidosis on $V_{\text{actin}}$
Abstract

Elevated levels of inorganic phosphate (P_i) are believed to inhibit muscular force by reversing myosin’s force-generating step. These same levels of P_i can also affect muscle velocity, but the molecular basis underlying these effects remains unclear. We directly examined the effect of P_i (30mM) 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 P_i to myosin’s ADP-bound state while changing the [ATP] probed rebinding to the rigor state. Surprisingly, the addition of P_i 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]. At pH 7.4 the effects of P_i were highly ATP-dependent, with P_i slowing V_{actin} at low ATP (<500uM), but a minor increase at 2mM ATP. The P_i-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 P_i-induced prolongation of the rigor state, which accounted for the slowing of V_{actin} at low ATP, and a P_i-induced detachment from a strongly-bound post-powerstroke state, which accounted for the increase in V_{actin} at high ATP. These findings suggest that P_i differentially affects myosin function: enhancing velocity if it rebinds to the ADP-bound state, while slowing velocity if it binds to the rigor state.

Keywords: Phosphate, Acidosis, Fatigue, Velocity
Introduction

Muscular force and motion are generated through the cyclical interaction of actin and myosin, in a process ultimately powered by the hydrolysis of ATP (Figure 1). In the 1980’s several investigations established that elevated levels of Pi reduced muscular force, leading to the notion that Pi-release by myosin was closely associated with force generation (12) and the rotation of the lever arm (2). This hypothesis postulates that Pi rebinds to myosin (M) in a state where 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.Pi state (32). While competing theories exist (3), models based on the Pi-induced detachment can account for much of the effect on muscular force (26). However, the effects of Pi 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 (Vus) (7) subsequent efforts revealed that Pi could induce either a minor increase in velocity, if the ATP level was high or a profound decrease in velocity at μM 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 Vus in muscle fibers. In agreement with the findings in muscle fibers (27), Pi was found to cause a mild increase in actin filament velocity (Vactin) at saturating ATP (14). Also in agreement with observations in muscle fibers, several authors reported that elevated levels of Pi decrease Vactin at μM 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 et al. (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 sub-saturating ATP is most accurately explained using a thermodynamic approach, where $V_{actin}$ is governed, not solely by detachment kinetics, but is also significantly influenced by the amount free energy available to myosin to drive filament motion (e.g. $\Delta G = -RT \ln [P_i][ADP]/[ATP]$). Raising $P_i$, they postulated, reduces $\Delta G$ 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 $\Delta G$), providing a rationale for the lack of an effect at mM 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 $P_i$
does not rebind to the AM.ADP state, but rather rebinds to the rigor state (AM) creating an AM.P_i state. This offered renewed support a detachment-limited explanation of the effects of P_i, reaffirming the notion that P_i slows \( \nu_{\text{actin}} \) by prolonging \( t_{\text{on}} \).

The equivocal findings at high ATP and the contrasting explanations for the P_i-induced depression in \( \nu_{\text{actin}} \) demonstrate that the mechanisms that underlie P_i-rebinding to myosin remain unclear. Given the close association between P_i and the generation of force and/or motion by myosin (12), a full understanding of the molecular basis of P_i-rebinding to myosin is crucial to attain a complete molecular level understanding of contraction.

A key unresolved issue is whether P_i rebinds to the AM.ADP state or the AM state (Figure 1). This has been a particularly challenging issue to address because skeletal muscle myosin has an inherently short ADP-lifetime when bound to actin (23). Fortuitously, our own recent work demonstrated that acidosis prolongs actomyosin’s ADP-bound state (AM.ADP) under the unloaded conditions of the motility assay (8). This finding provides a unique opportunity to determine if P_i can rebind to the ADP state under these conditions and what effects it might have of myosin function. Therefore, by systematically altering the pH, ATP and P_i concentrations we manipulated whether P_i was able to bind to the AM.ADP state or the rigor state. The findings might also provide insight into muscular fatigue because elevations in both P_i and H^+ are believed to play a role by directly affecting the actomyosin interaction.

We found that the effects of P_i on myosin were dependent on both ATP and pH. At pH 7.4, elevated P_i depressed \( \nu_{\text{actin}} \) below 1mM ATP and caused a mild increase in \( \nu_{\text{actin}} \) above 2mM ATP. However, as the pH was lowered, the depression of \( \nu_{\text{actin}} \) at low
ATP was minimized (pH 6.8) and even reversed (pH 6.5). Furthermore the P_i-induced increase in $V_{\text{actin}}$ observed at 2mM ATP became significantly more pronounced as the pH was decreased. Fitting the data to a mechanistic kinetic model required the addition of P_i-bound rigor state (AM.P_i) and a P_i-induced detachment from the AM.ADP state.

**Methods**

**Proteins**

All animal tissue was obtained in accordance with University and NIH policies. 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 (MHC) and both the regulatory (RLC) and essential light chains (ELC) 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 MHC (4).

Following purification, the myosin was diluted in 50% glycerol (vol/vol) and stored at -20°C. An additional purification was done on the day of an experiment by adding an equimolar amount of filamentous actin and 2mM ATP and then centrifuging the sample at 400,000g for 20min. 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. In order to stabilize the actin in filamentous form and visualize it in epi-fluorescence, phalloidin with tetramethylrhodamine isothiocyanate (TRITC) (Sigma-Aldrich Inc., St.
Louis, MI) was added to 1uM 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 (~30mg/ml) in 50% glycerol to 200µg/ml in myosin buffer (MB, composed of 300mM KCl, 25mM Imidazole, 1mM EGTA, 4mM MgCl₂, 1mM DTT). Following the final purification step the myosin was further diluted to 100µg/ml in myosin buffer and added to the nitrocellulose coated microscope coverslip.

Subsequently, every step in the preparation of the flow cell utilized a form of actin buffer (AB), composed of 25mM KCl, 25mM Imidazole, 1mM EGTA, 4mM MgCl₂). 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 concentration in each buffer to maintain a constant total ionic strength of 125mM. The constituents of all motility buffers were calculated using WinMax Chelator with the stability constants provided within the program (29). 1% methylcellulose and an oxygen scavenging system (1725.5mg glucose, 7.5mg glucose oxidase and 1.35mg catalase, in 600µL ddH₂O) 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 0mM Pi condition that the [Pi] was actually ~0.5mM due to contamination and ongoing hydrolysis (7). In addition, we also assumed 0.025% contaminating pyrophosphate (PP₃), which has a higher affinity for myosin than Pi (1).
However, recent evidence indicates that this amount of PPi contamination has no
discernable effect on myosin function in an in vitro motility assay (1).

In vitro motility assay

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 (1uM unlabeled actin in AB) was applied to prevent
any remaining rigor-like myosin molecules from retarding V_{actin}. This was followed by a
wash with 1mM ATP in AB to release any actin bound to active myosin molecules. The
flow cell was then washed with AB without ATP and then TRITC-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 motility buffer enabled
motion as it included the desired amount of ATP as well as the desired Pi and pH.

The temperature of the flow cell was maintained at 30°C using an objective
temperature controller (20/20 Technologies Inc. Wilmington, NC). Fluorescent actin
filament motion was visualized using an ICCD camera (model XR/MEGA-10EX™ S30,
Stanford Photonics, Inc. Palo Alto, CA, USA) mounted to a Nikon Eclipse Ti® inverted
microscope with a 100X, 1.4NA CFI Plan Apo objective. Video was captured by
coupling the ICCD camera to an Epix-LVDS frame grabber (Epix, Inc., Buffalo Grove,
IL, USA). The camera and frame grabber were controlled by a PC using PIPER
Control™ software (Stanford Photonics, Inc. Palo Alto, CA, USA). Three, 30s 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**

Actin filament velocity was manually tracked using the MTrackJ plug-in for ImageJ (NIH Image). Typically, the paths of eight individual filaments were tracked for each 30s 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, were compiled and then used for statistical comparisons.

All filaments under each level of pH and Pi, at 2mM ATP, were analyzed using a two-way ANOVA (pH x Pi) with the α-level set at p<0.05 in SigmaStat® (Systat Software San Jose, CA). The ATP curves were fit with a Michaelis–Menten relationship where:

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

with \(V_{\text{max}}\) the maximal \(V_{\text{actin}}\) at infinite [ATP] and \(K_M\) the [ATP] at which \(V_{\text{actin}}\) is \(\frac{1}{2}\) \(V_{\text{max}}\). The analysis was performed using SigmaPlot 8.0® (Systat Software San Jose, CA) employing a least-squares fit.

**Results**

As expected, at 2mM ATP \(V_{\text{actin}}\) progressively decreased with increasing levels of acidosis, (Figure 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 30mM 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 30mM 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 x 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**

In order to investigate the kinetic basis of these observations $V_{\text{actin}}$ was measured as a function of [ATP], pH and [P_i] (Figure 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 $V_{\text{max}}$ at both pH 6.8 and 6.5 compared to 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 below 500uM ATP. Due to this issue the curve is poorly constrained in this region and therefore estimates of $K_M$ should be interpreted cautiously.

As expected, elevating P_i depressed $V_{\text{actin}}$ at sub-saturating 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 (Figure 3). This P_i-induced enhancement of $V_{\text{actin}}$ at pH 6.5 was particularly striking at 300µM ATP, where myosin was completely incapable of moving actin in the absence of
P$_i$, but moved the actin filaments at near the maximum value under these conditions (3.2 µm·s$^{-1}$) in the presence of high P$_i$.

The increases at pH 7.4, although significant at 2 and 4mM ATP, were small (~8-10%) and not consistently observed at all ATP levels, for example 5mM 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 P$_i$ at pH 6.5 resulted in a decreased K$_M$.

Kinetic analysis

Double reciprocal plots of the V$_{actin}$ vs. ATP data enabled a simple kinetic analysis to determine the effect of P$_i$ 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$_{actin} \approx d/t_{on}$. In this model, the duration of strong-actin binding can be further dissected into the time required for ADP-release (t$_{ADP}$) by myosin, plus the time required for ATP to bind and cause dissociation from actin (t$_{rigor}$)(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 2$^{nd}$ order ATP-dissociation (k$_{+ATP}$) constants under each condition (Figure 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\(^{-1}\) is similar to previous estimates from single muscle fibers (38) and \textit{in vitro} motility experiments (15). In addition, the \(k_{+\text{ATP}}\) of \(1.85 \times 10^6\text{M s}^{-1}\), 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 in half from pH 7.4 to 6.8 and a further, almost three-fold decrease at pH 6.5 (Table 1). In contrast, added \(P_i\) 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 \(P_i\), at 6.8 and tripled at pH 6.5.

\(k_{+\text{ATP}}\) demonstrated a differential response when pH alone was manipulated, increasing slightly at pH 6.8 compared to pH 7.4 but slowing at pH 6.5. In contrast, elevated \(P_i\) caused \(k_{+\text{ATP}}\) to decrease at each level of pH. This result suggests that elevated levels of \(P_i\) concentrations significantly depress the rate of \(k_{+\text{ATP}}\) of myosin. This is consistent with the idea that \(P_i\) readily competes with ATP for binding to myosin’s active site (1; 27).

**Discussion**

We have used the \textit{in vitro} motility assay to probe the molecular basis of the effects of \(P_i\) on the actomyosin interaction. We found that the effects of \(P_i\) on \(V_{\text{actin}}\) are dependent on both ATP and pH, with elevated \(P_i\) depressing \(V_{\text{actin}}\) 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 $P_i$ increases the $K_M$, due to the $P_i$-induced decrease in $V_{\text{actin}}$ at low ATP (Figure 3, Table 1). In a simple detachment-limited model of $V_{\text{actin}}$ (i.e. $V_{\text{actin}} = d/t_{\text{on}}$), this decrease in $V_{\text{actin}}$ could be due to either a decrease in myosin’s step size ($d$) and/or an increase in the duration of strong actin-binding ($t_{\text{on}}$) (16). Previous single molecule measurements in the laser trap assay suggest that $d$ is unaffected by either high levels of $P_i$ (2) or by low pH (8), at high levels of ATP. This, therefore, suggests that the $P_i$-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 ($\mu$M levels), the duration of the rigor-lifetime dominates $t_{\text{on}}$ (2), therefore the depressive effects of $P_i$ on $V_{\text{actin}}$ observed at low ATP are likely due to an effect of $P_i$ on the rigor state. A simple explanation for this observation is that $P_i$ rebinds to actomyosin in the rigor state, prolonging its lifetime through the formation of an AM.P$_i$ 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 $P_i$-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). We then used this model to simulate fits to the $V_{\text{actin}}$ vs. ATP (Figure 5). These simulations revealed that the inclusion of an AM.P$_i$ state was required to reproduce the $P_i$-dependence of $K_m$ observed pH 7.4 and 6.8. We used this same model to probe the molecular basis of the $P_i$-induced increase in $V_{\text{actin}}$; however, the addition of an AM.P$_i$ state to the simple detachment-limited model could not capture this aspect of the data (Figure 5a), this was especially
evident at low pH (Figure 5a). Indeed, capturing this aspect of the data required the inclusion of a P_i-induced detachment from a post powerstroke, AM.ADP state (Figure 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 (Figure 5b). Therefore the inclusion of both modifications of the model required to fully reproduce both the depressive effect of P_i at low ATP and the enhancement of $V_{\text{actin}}$ at high ATP (Figure 5c). This suggests a novel view of how P_i 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 P_i, this modified detachment-limited model provides a simple mechanistic explanation for our data.

**Relevance to previous work**

Our observation that P_i 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 P_i 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 P_i can readily rebind to myosin’s empty nucleotide binding pocket. However, our data also suggest that P_i can, under very different conditions, rebind to the myosin in an AM.ADP state.
$P_i$ can rebind to myosin in the AM.ADP state

Our own previous work, using a single molecule laser trap assay, indicates that acidosis prolongs $t_{on}$ by prolonging the lifetime of the AM.ADP state with little effect on the ATP-dissociation 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, $t_{on}$ is dominated by the AM.ADP lifetime, thus it is this state that $P_i$ likely rebinds to under these conditions.

This conclusion directly contradicts the suggestion by Amrute-Nayak et al. that the rebinding of $P_i$ to the AM.ADP state is highly unfavorable under the unloaded conditions of a motility assay (1). However, our contradictory conclusions may simply be the result of the differing conditions between the two assays. Amrute-Nayak et al. (1) examined the effects of $P_i$ rebinding to myosin at pH 7.0 and nM levels of ATP, conditions that strongly favor rebinding to the rigor state over the AM.ADP state. And as noted above we see exactly the same depressive effect of $P_i$ under similar conditions in the present study (Figure 3a). However at low pH and high ATP, where $t_{on}$ is likely dominated by the AM.ADP lifetime, we observe the opposite effect on $V_{actin}$ suggesting that $P_i$ can readily rebind to the AM.ADP state even under unloaded conditions. In fact these conditions, under which $t_{on}$ predominantly consists of the AM.ADP lifetime, may be more similar to those experienced under high load where the load dependent nature of ADP-release prolongs the duration of the AM.ADP state (35). Under these loaded conditions it is generally believed that $P_i$ reduces force by rebinding to and causing the dissociation of actin and myosin from the AM.ADP state (12). Therefore our data are not
necessarily inconsistent with the observations or conclusions of Amrute-Nayak et al. (1) but rather suggest that $P_i$ can indeed rebind to the AM.ADP state when it is the dominant strongly bound state.

**Mechanism of the $P$-induced increase in $V_{actin}$**

The subtle $P_i$-induced increase $V_{actin}$ we observed at high ATP at pH 7.4, is both qualitatively and quantitatively consistent with previous observations of both unloaded shortening velocity in muscle fibers (27; 28) and $V_{actin}$ in a motility assay (14). However, the much more pronounced increases in $V_{actin}$ at low pH and high ATP were surprising and much greater than observed in any previous studies. Interestingly, Greenberg et al. (9) recently reported a strikingly similar increase in $V_{actin}$ in response to a similar increase in $P_i$ under acidic conditions. They, however, did not attribute the effects to $P_i$ because they manipulated other constituents of the assay in addition to $P_i$, mostly 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 $P_i$.

This increase in $V_{actin}$ in response to $P_i$, is also difficult to reconcile with the mechanism proposed by Hooft et al. (15), who suggested that increasing $P_i$ lowers $V_{actin}$ by affecting the free energy ($\Delta G$) available to drive actin filament motion. However, by raising $P_i$ and lowering pH, in the present study, we presumably decreased $\Delta G$ 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 $V_{\text{actin}}$ are more complex in our assay due to the additional manipulation of pH.

One potential mechanism to explain the increased $V_{\text{actin}}$ comes from the double reciprocal plots of our $V_{\text{actin}}$ vs ATP data (Figure 4) which suggests that $P_1$ accelerates the rate of ADP-release from myosin (Figure 4, Table 1). However, the presence of additional states and transitions, necessary in our model simulations, mean that the assumptions of this kinetic analysis may be invalidated. For example, if $P_1$ rebinding causes dissociation of myosin from actin before it progresses through ADP release and rigor it would negate the notion that $t_{\text{on}}$ 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 $P_1$-induced detachment from a strongly-bound actin state rather than an actual speeding of the ADP-release rate.

In fact, a $P_1$-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 $P_1$ on force in muscle fibers. However, their mechanism implies that $P_1$-rebinding also reverses the rotation of the lever (32), a fact that is difficult to reconcile with the $P_1$-induced increase in $V_{\text{actin}}$. Reversal of the lever arm rotation would also reverse the previous displacement of the actin filament. In a simple detachment-limited model where $V_{\text{actin}} = d/t_{\text{on}}$ this predicts that elevated $P_1$ would decrease rather than speed $V_{\text{actin}}$. This therefore leads us to suggest that $P_1$ rebinding does not necessarily reverse myosin’s powerstroke but rather induces detachment from actin in a post-powerstroke state. In this case, since the displacement of the actin filament from myosin’s powerstroke is maintained while $t_{\text{on}}$ is decreased $V_{\text{actin}}$ increases.
This proposed mechanism creates a situation where the release of $P_i$ 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 suggests that the release of $P_i$ and ADP can indeed occur quite rapidly off actin under certain conditions. For example, elevated levels of $P_i$ 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 $P_i$ and ADP from myosin while off actin (19) to accurately fit the ATPase results. For a structural viewpoint, it was suggested that since $P_i$-induced detachment occurs following a weak to strong transition the active site remains in a configuration that favors the rapid release of both $P_i$ and ADP.

$P_i$ reduces myosin’s duty ratio at high ATP

One prediction of the proposed mechanism that $P_i$ 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 $P_i$ of myosin’s ATPase rate in fibers (30; 31). Therefore to test this prediction of our model we measured the effect of $P_i$ on myosin’s duty ratio at 2mM ATP. This was done using the method of Uyeda et al. in which $V_{\text{actin}}$ is determined at limiting amounts of myosin.
As predicted by the model, elevated P$_i$ reduced the estimate of myosin’s duty ratio. At pH 7.4, P$_i$ reduced the duty ratio from 3.5 to 2.8% (Figure 6). The P$_i$-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 P$_i$ can induce detachment from the post powerstroke AM.ADP state.

**Conclusions**

In the *in vitro* motility assay we have demonstrated that elevated levels of P$_i$ can have distinctly different effects on $V_{\text{actin}}$ depending on ATP and pH; slowing $V_{\text{actin}}$ at low ATP and high pH, while having the opposite effect at high ATP and low pH. These findings suggest that P$_i$ 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 $V_{\text{actin}}$ at low ATP can be explained in a model where P$_i$ competes with ATP to form an AM.P$_i$ state. In contrast, the enhancement of $V_{\text{actin}}$ can be explained by a model in which P$_i$ induces detachment from a post powerstroke AM.ADP state.

The structural correlates of these P$_i$-bound states are unclear, however the known structural differences between the AM.ADP and AM states (13) may impact how P$_i$ 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 P$_i$-rebinding to the active site.
and impact the functional effects on actomyosin motility. Related to this idea, recent molecular dynamics simulations suggest that there may be multiple pathways of P\textsubscript{i}-release from the active site (5). This leads to the speculation that the rebinding of P\textsubscript{i} 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 P\textsubscript{i} can rebind to myosin in more than one biochemical state might indicate that the rebinding of P\textsubscript{i} to myosin’s active site is more promiscuous than previously believed. Thus the internal atomic motions within the active site that govern P\textsubscript{i} 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 P\textsubscript{i}-release and rebinding within myosin’s active site to provide a more detailed picture of how P\textsubscript{i} is coupled to the generation of force and motion during contraction. Since our conditions of high P\textsubscript{i} 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 P\textsubscript{i} and H\textsuperscript{+} 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 P\textsubscript{i}-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.
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Figure Legends

**Figure 1.** A schematic model of myosin’s cross-bridge cycle that links the biochemical and mechanic events. \(d\) refers to myosin’s unitary displacement and \(t_{\text{on}}\) refers to the duration of strong actomyosin binding. \(t_{\text{ADP}}\) and \(t_{\text{rigor}}\) refer the duration of the AM.ADP and AM states respectively(24). Putative states of \(P_i\) re-binding are indicated with red arrows.

**Figure 2.** Effect of \(P_i\) and pH at 2mM ATP. Data represents mean ± SD, n ranges from 27 to over 100 filaments. Data were analyzed using a two-way ANOVA (pH x \(P_i\)). Significance (*) was set at \(p<0.05\) for the main effect and the interactions (#)

**Figure 3.** \(V_{\text{actin}}\) vs. ATP. Data points are means ± 95% C.I. Data points without added \(P_i\) are black circles with corresponding line fits the solid black lines. Data points for 30mM added \(P_i\) are gray squares and corresponding fits are dashed gray 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\(^{-1}\) for \(V_{\text{max}}\) and 372 ± 40 and 773 ± 86 μM for \(K_m\) 0 and 30mM \(P_i\) respectively. At pH 6.8 \(V_{\text{max}}\) was 3.2 ± 0.1 and 5.6 ± 0.2 μm s\(^{-1}\) and \(K_m\) 119 ± 24 and 687 ± 55 μM at 0 and 30mM \(P_i\) respectively. At pH 6.5 \(V_{\text{max}}\) was 2.2 ± 0.1 and 3.9 ± 0.1 μm s\(^{-1}\) and \(K_m\) 821 ± 192 and 202 ± 28 μM at 0 and 30mM \(P_i\) respectively.

**Figure 4.** Double reciprocal of ATP curves. Data points without added \(P_i\) are black circles with corresponding line fits the solid black lines. Data points for 30mM added \(P_i\) are gray squares and corresponding fits are dashed gray lines. \(V_{\text{actin}}\) was divided by a \(d\) value of 10 nm (10) to estimate \(t_{\text{on}}\). This was plotted against 1/ATP and fit to best fit using the equation \(t_{\text{on}} = 1/k_{-\text{ADP}} + 1/k_{-\text{ATP}}[\text{ATP}]\). Values represent mean ± 95% C.I. The estimates for the ADP-release rate (\(k_{-\text{ADP}}\)) and (\(k_{-\text{ATP}}\)) under each condition are displayed in Table 1.

**Figure 5.** *In silico* fits to experimental data. Fits to the data from computer simulations using a mechanistic kinetic model (see Supplemental Material). The \(P_i\) data points are gray squares with corresponding fits are the dashed gray lines, while the data points in the absence of \(P_i\) are black circles and corresponding line fits in black. The fits in Figure 5a include an AM.Pi state but no rebinding to and dissociation from a post-powerstroke AM.ADP state. Figure 5b are our fits to the data without an AM.Pi state but include \(P_i\)-induced dissociation from AM.ADP in a post-powerstroke state. The fits in 5c include the addition of both an AM.Pi state and a \(P_i\)-induced post-powerstroke dissociation from AM.ADP state.

**Figure 6.** Effect of \(P_i\) and acidosis on myosin’s duty cycle. Estimates of myosin’s duty (\(f\)) cycle were made by measuring \(V_{\text{actin}}\) as a function of the surface concentration of myosin, ranging from 5 to 100μgml. For comparisons \(V_{\text{actin}}\) was normalized to the maximum value observed under each condition. Filament length was measured using
ImageJ (NIH) and converted to a number of available heads ($N$) using estimates of myosin density contained within Harris and Warshaw (11). Therefore each data point represents the velocity of a single filament and its corresponding length. The data were fit using to the equation of Uyeda et al. (33) to derive estimates of the duty cycle, $V_{\text{actin}} = (V_{\text{max}}) x (1-(1-f)N)$: where $V_{\text{max}}$ the extrapolated maximal $V_{\text{actin}}$, $f$ the duty ratio and $N$ the number of myosin heads capable of interacting with an actin filament. The estimate of duty cycle under control conditions (pH 7.4, no added $P_i$) was 3.5%, similar to previous estimates under similar conditions (11). The addition of $P_i$ at pH 7.4 decreased $f$ to 2.8% with a further reduction to 2% at pH 6.5.
Table legend.

Table 1. Derived rate constants for ADP-release ($k_{ADP}$) and ATP-induced dissociation ($k_{ATP}$). Values are means with 95% confidence intervals. * indicates a significant (p<0.05) effect of 30mM $P_i$. † indicates a significant effect of pH alone.
Figure 2

The graph shows the effect of pH on the velocity of actin ($V_{actin}$) at concentrations of 0 mM and 30 mM Pi. The velocity is measured in micrometers per second ($\mu$m/s). The pH values tested are 7.4, 6.8, and 6.5. The graph indicates a significant reduction in velocity as the pH decreases from 7.4 to 6.5.

- At pH 7.4, the velocity for both 0 mM and 30 mM Pi is high.
- At pH 6.8, the velocity for 30 mM Pi is significantly lower than that for 0 mM Pi.
- At pH 6.5, the velocity for both conditions is significantly reduced compared to the other pH values.

Significance markers are used to indicate statistical differences.

* indicates a significant difference compared to 0 mM Pi at the same pH.
# indicates a significant difference compared to the previous pH value.

The graph emphasizes the impact of pH on actin velocity in the presence of Pi.
Figure 3
Figure 4

1/ATP (mM) vs. $t_{on}$ (ms) or $d/V_{actin}$ for pH 7.4, pH 6.8, and pH 6.5.
<table>
<thead>
<tr>
<th>pH</th>
<th>[P_i] mM</th>
<th>$k_{\text{ADP}}$ (s^{-1}) (95% C.I.)</th>
<th>$k_{\text{ATP}}$ (x 10^6 M^{-1} s^{-1}) (95% C.I.)</th>
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<td>653 (598 - 718)</td>
<td>1.85 (1.78 - 1.92)</td>
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<td>0.99 (0.96 - 1.03) *</td>
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<tr>
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<td>0</td>
<td>287 (277 - 297)*</td>
<td>2.87 (2.57 - 3.72)†</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>605 (378 - 452)*</td>
<td>1.20 (1.07 - 1.37)*</td>
</tr>
<tr>
<td>6.5</td>
<td>0</td>
<td>132 (143 - 199)*</td>
<td>0.36 (0.26 - 0.56)†</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>456 (338 - 384)*</td>
<td>0.18 (0.17 - 0.19)*</td>
</tr>
</tbody>
</table>

Table 1.