In muscle inorganic phosphate strongly decreases force generation in the presence of millimolar MgATP, whereas phosphate slows shortening velocity only at micromolar MgATP concentrations. It is still controversial whether reduction in shortening velocity by phosphate results from phosphate binding to the nucleotide-free myosin head or from binding of phosphate to an actomyosin-ADP state as postulated for the inhibition of force generation by phosphate. Because most single-molecule studies are performed at micromolar concentrations of MgATP where phosphate effects on movement are rather prominent, clarification of the mechanisms of phosphate inhibition is essential for interpretation of data in which phosphate is used in single molecule studies to probe molecular events of force generation and movement. In *in vitro* assays we found that inhibition of filament gliding by inorganic phosphate was associated with increased fragmentation of actin filaments. In addition, phosphate did not extend dwell times of Cy3-EDA-ATP (2′(3′)-O-[[2-[[6-[[2-[[3-[(1-ethyl-1,3-dihydro-3,3-dimethyl-5-sulfo-2H-indol-2-ylidene)-1-propenyl]-3,3-dimethyl-5-sulfo-3H-indolio]]-1-oxohexyl]amino]ethyl]carbamoyl]ATP) but reduced the number of Cy3-signals per field of view, approaching 50% at phosphate concentrations of 1–2 mM. Apparently, inhibition of movement does not result from binding of phosphate to an actomyosin-ADP intermediate as proposed by Hooff and coworkers (Hooff, A. M., Maki, E. J., Cox, K. K., and Baker, J. E. (2007) *Biochemistry* 46, 3513–3520) but, rather, from forming a strong-binding actomyosin-phosphate intermediate.

Muscle contraction involves the sliding of actin filaments relative to myosin filaments (1, 2), which is driven by cyclic interactions of the myosin head domains with the actin filaments, powered by hydrolysis of ATP. During each ATP-hydrolysis cycle chemical energy of ATP hydrolysis is transformed into mechanical work by a multistep power-stroke which drives the actin filaments several nanometers past the myosin filaments. The power-stroke is associated with the release of the ATP hydrolysis products, Pi (inorganic phosphate) and ADP, from the active site (3). It is generally believed that Pi release from the AM-ADP-Pi\(^4\) complex is closely related to the initiation of the power-stroke (4) and to the transition of the myosin head domain from states of weak and non-stereospecific actin binding to states of strong and stereospecific binding to actin (5–7). Because of the assumed close relation between power stroke and the release of inorganic phosphate from the myosin head domain, studying the effects of inorganic phosphate on contracting muscle fibers became a main element to elucidate the relation between P\(_i\) release and force generation. Effects of P\(_i\) were examined on isometric force (4, 8–14) and unloaded shortening velocity (9, 13, 15) as well as on force transients in response to release of P\(_i\) from caged P\(_i\) (16, 17). As a result of these studies it was proposed that inhibition of active force by inorganic phosphate results from rebinding of P\(_i\) to the AM-ADP intermediate that is formed after P\(_i\) release (cf. Scheme 1). Thus, the release of phosphate is reversed and a strong binding force generating cross-bridges in the AM-ADP state are reversed to a weak binding non-force generating AM-ADP-Pi state (the AM-ADP-Pi\(^4\) state in Scheme 1) that is in rapid equilibrium with the detached M-ADP-Pi\(^1\) intermediate. To fully account for the observed steady state kinetic data and the force transients recorded upon release of P\(_i\) from caged P\(_i\), it was proposed that a strong binding AM-ADP-Pi intermediate (the AM-ADP-Pi\(^4\) state in Scheme 1) must exist in the path from non-force-generating M-ADP-Pi\(^1\)/AM-ADP-Pi\(^1\) states to the force generating the AM-ADP state (16–18). In recent work using the P\(_i\) analog AlF\(_4^-\), evidence for such a strong binding intermediate in the active site has been presented (19).

Some results, however, appeared unexplained by this concept. First, at low ATP concentrations an increase in inorganic phosphate (P\(_i\)) to 50 mM caused a significant decrease in unloaded shortening velocity (20). Second, when fiber stiffness of isometrically contracting fibers was measured at different speeds of stretch, the stiffness-speed relations recorded in the presence of 5–10 mM P\(_i\) were shifted to slower stretch velocities (21). Based on these observations it was proposed that, as previously shown in solution studies (22–24), inorganic phosphate may also bind to the empty nucleotide binding pocket, i.e. to the AM state in Scheme 1, thus competing with ATP for the active site by forming an AM-Pi\(_4\)-complex. Analogous to the effect on unloaded shortening velocity, the sliding velocity of actin filaments driven by muscle myosin in *in vitro* motility assays was...
also found slowed down by inorganic phosphate at low MgATP concentrations (25). As one possible mechanism, these authors also proposed competitive inhibition of ATP-binding to the nucleotide-free myosin head by inorganic phosphate, assuming that P_i may bind to the nucleotide free active site, forming an AM-P_i complex. As an alternative mechanism, these authors proposed that inorganic phosphate, before ADP release, may bind to a secondary site on the myosin head that is not the site from which P_i is released after cleavage of ATP. The thus-formed AM-ADP-P_i state was thought to exert a resistive load against the remaining motion generating myosin heads until P_i and ADP are released and binding of ATP changes the myosin head back to a weak binding AM-ATP intermediate. In more recent work (26), however, it was proposed that binding of P_i to the AM-ADP state, just as postulated for the suppression of isometric force by P_i, also causes reduced actin filament sliding velocity. For this to work, Hooyt et al. (26) proposed that a fixed resistive load, generated by cross-bridges in “motion-resisting” conformations/states, acts against the driving force generated by heads in “driving” conformations/states. Because sliding velocity at low MgATP also showed a logarithmic dependence on the concentration of P_i, as described for isometric force in fibers (14), Hooyt et al. (26) proposed that P_i does not affect the population of cross-bridges that generate the resistive load but that, rather, the population of driving heads is reduced when P_i is raised.

Thus, it is still controversial by which mechanism inorganic phosphate inhibits gliding velocity in motility assays and unloaded shortening velocity in fibers, i.e. by binding to the AM-ADP intermediate or by binding to the nucleotide-free AM state forming an AM-P_i intermediate that inhibits gliding of actin filaments along myosin. We, therefore, addressed this question using in vitro gliding and single molecule dwell-time assays.

We first reexamined the effect of inorganic phosphate on the gliding velocity of actin filaments on a myosin-coated surface (ensemble assay) to ensure that our experimental conditions reproduce previous results (25). Using Cy3-EDA-ATP as a fluorescently labeled substrate of myosin II (27–29), we measured the dwell time of bound nucleotide and the number of fluorescent signals per field of view. This allowed us to test distinct predictions of the different mechanisms proposed for inhibition of actin filament gliding velocity by P_i. In the presence of P_i, actin filament gliding velocity was reduced at low but not at saturating MgATP concentrations, as previously described. The nucleotide dwell time, probed with Cy3-EDA-ATP, and remained unchanged upon the addition of 5 mM P_i, whereas the number of Cy3 signals per field of view in the presence of 5 mM Cy3-EDA-ATP was reduced to 50% in the presence of 1–2 mM phosphate. These data are consistent with concepts in which actin filament gliding velocity is reduced by binding of P_i to the empty nucleotide binding pocket of myosin, thus increasing the resistive load against filament gliding when P_i is increased. Preliminary results have been presented in abstract form (30).

**EXPERIMENTAL PROCEDURES**

**Buffer—**Myosin extraction buffer contained 500 mM NaCl (Merck), 10 mM HEPES, 5 mM MgCl_2, 2.5 mM MgATP, and 30 mM DTT, pH 7. Actin polymerization buffer was composed of 100 mM KCl (Merck) and 10 mM HEPES, pH 7. Assay buffer (AB) contained 25 mM imidazole hydrochloride, 25 mM NaCl, 4 mM MgCl_2, 1 mM EGTA, 10 mM dithiothreitol, and an oxygen scavenging system of 18 μg/ml catalase, 0.1 mg/ml glucose oxidase, 3 mg/ml d-glucose (Merck), pH 7.4.

For experiments in which different MgATP concentrations were used or when inorganic phosphate or αIF_4 was added, the basic buffer composition was equivalent to the AB buffer, and the ionic strength was maintained at 55 mM by adjusting NaCl concentration in the AB buffer accordingly. To remove contaminant pyrophosphate (PP_i), the pH of the 100 mM NaH_2PO_4 stock solution was set to 9.0, and the stock solution was autoclaved at 120 °C for 15 min. To avoid precipitation, αIF_4-containing solutions were prepared immediately before use by the addition of Al(NO_3)_3 (Merck) and NaF (Fluka) in a 1:5 ratio. The pH was adjusted to 7.0. All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

**Proteins—**Fast skeletal muscle myosin was extracted from skinned fibers of rabbit psoas muscle as described previously (31). First, muscle fiber bundles were isolated from the psoas muscle and prepared for long time storage in liquid nitrogen (32). For extraction of myosin, fiber bundles were thawed in skinnning solution without sucrose and allowed to equilibrate for 1 h. Single muscle fibers of 1–1.5 cm in length and about 100 μm in diameter were isolated (33). For in vitro motility assays myosin was extracted from a single fiber by incubation in 5 μl of myosin extraction buffer at 1–2 °C for 30 and 10 min for dwell-time assays. To minimize content of denatured myosin, myosin was extracted from fibers isolated from freshly thawed bundles, and the extract was used within 8 h after extraction. Actin was purified from rabbit back muscle as previously described (34). TRITC-labeled F-actin was produced by polymerizing G-actin in the presence of TRITC-phalloidin (35). Actin filaments decorated with full-length myosin molecules in the nucleotide-free rigor state were formed by adding 10 mM EDTA to a mixture of F-actin and myosin extract to chelate Mg^{2+} of the myosin.
extracted F-actin was adjusted to obtain an ~20:1 molar ratio of actin monomers to myosin molecules. By this procedure myosin not bound to actin was kept to a minimum. After 30 min of incubation at 0 °C, the solution was diluted ~150-fold with AB buffer to obtain a suitable concentration of decorated actin filaments. This final solution was then applied to the flow cell. The myosin-decorated actin filaments were firmly immobilized on the BSA-coated surface, presumably by nonspecific binding of the full-length myosin (31). To locate decorated actin filaments in dwell-time experiments with Cy3-EDA-ATP, F-actin was polymerized in the presence of Alexa-488-phalloidin instead of TRITC-phalloidin.

Total Internal Reflection Fluorescence Microscopy—For all experiments an objective type total internal reflection fluorescence microscope was used. This was arranged in an inverted configuration on an optical table using a 60x oil immersion objective lens with high numerical aperture (Plan Apo 60x, NA 1.45, Oil; Olympus, Japan). The beam of a green laser (diode pumped, frequency doubled Nd:YAG laser, DPSS 532–50, Coherent, Palo Alto, CA) and of an air-cooled argon ion laser (Type 532-AP-AO1, Melles Griot) were used to excite TRITC-phalloidin, Cy3-labeled nucleotides, and Alexa-488-phalloidin. Emitted light was imaged by a tube lens onto the active area of the photocathode of an image intensifier (VS4–1845; Video Scope International, Sterling, VA). The image intensifier formed an intensified image on the active area of a CCD camera (ORCA-ER digital CCD camera C4742–80-12AG, Hamamatsu Photonics). For camera control and data acquisition, the Simple PCI software (Compix, Inc.) was used. Further details are provided in the supplemental material.

In Vitro Gliding Assay—The in vitro gliding assay was modified from the assay previously described (37). The gliding assay was performed in a flow cell by the following steps (31). The glass surface of the flow cell was coated with BSA (AB buffer containing 0.5 mg/ml BSA). This was followed by infusion of 5 μl of the myosin extraction solution. Adsorption of myosin to the surface was allowed for 30 s and stopped by infusion of 2 × 5 μl of AB buffer; subsequently, 5 μl of solution with non-fluorescently labeled F-actin was introduced to block denatured myosin followed by incubation with AB buffer containing 2 mM MgATP to allow dissociation of the unlabelled actin from native myosin heads. A subsequent double wash with 2 × 5 μl of AB buffer served to remove MgATP. Next, the flow cell was filled with a solution containing 5 mM TRITC-phalloidin-labeled F-actin and subsequently washed with 4 × 5 μl of AB buffer to remove free TRITC-phalloidin. Finally, translocation of actin filaments was started by application of AB buffer containing various concentrations of MgATP and 0.5 mg/ml BSA. Inorganic phosphate or AlF₄⁻ was added to the AB buffer, and ionic strength was adjusted to 55 mM. Experiments were performed at room temperature (22–25 °C). Further details are provided in the supplemental material.

A Dwelt-time Assay and Number of Events per Field of View—Cy3-EDA-ATP, a fluorescent ATP analog in which the ribose is labeled at the 2’ and 3’ position was used as a fluorescently labeled substrate for myosin to follow binding and dissociation of nucleotide (27–29). The Cy3-EDA-ATP used was a mixture of the 2’ and 3’ Cy3-EDA-ATP isoforms. For recording of dwell times of fluorescent nucleotide, flow cells were coated with AB buffer containing 0.5 mg/ml BSA. Then solutions with decorated actin filaments were injected into the flow cell. After incubation for 1 min, unbound protein was washed out with AB buffer. Finally, 5 nM Cy3-EDA-ATP in AB buffer, including the oxygen scavenging system, was added. Fluorescent signals of Cy3-EDA-ATP were observed as the appearance of discrete spots in the total internal reflection fluorescence microscope when Cy3-EDA-ATP bound to myosin. Final concentrations of myosin and actin in the flow cell were 100 pm and 2 nm, respectively. Further details of experimental conditions and procedures to optimize selection of specific binding events of individual Cy3-EDA-ATP molecules are described in the supplemental material.

Gliding velocity and dwell-time analysis was done using custom-written software based on Mathematica (Wolfram Research, Inc.). The analysis routines are explained in the supplemental material.

RESULTS

Effect of Inorganic Phosphate on Actin Filament Gliding Velocity—First, we examined the effect of inorganic phosphate on the velocity of actin filament gliding on a myosin-coated surface to reproduce previous data (25). Gliding velocity of actin filaments was measured at MgATP concentrations ranging from 30 μM to 5 mM both in the absence and presence of 5 mM phosphate. In the absence of Pᵢ, filament gliding velocity increased with MgATP concentration and reached MgATP-independent values around 2 μM MgATP (Fig. 1). In the presence of 5 mM Pᵢ, actin filament gliding velocity was reduced at MgATP concentrations below 200 μM. For MgATP concentrations >0.5–1 mM, effects of 5 mM Pᵢ were no longer statistically significant.

Actin Filaments Tend to Fragment in the Presence of Pᵢ but Only at Low MgATP Concentrations—Although the inhibitory effect of inorganic phosphate on actin gliding velocity was fully reversible, to our surprise actin filaments in the motility assay tended to break in the presence of inorganic phosphate but only at the low MgATP concentrations (Fig. 2). Quantification of this effect (Fig. 2B) shows that 100 s after starting filament gliding by the addition of MgATP, filament length was essentially unchanged at 2 μM MgATP plus 5 mM Pᵢ and at 30 μM MgATP but without Pᵢ. At 30 μM MgATP plus 5 mM Pᵢ, in contrast, average filament length is reduced to about 50% by progressive fragmentation of actin filaments. This observation may not be unexpected if reduction in gliding velocity by Pᵢ at low MgATP concentrations (Fig. 1) results from an additional resistive load that acts on actin filaments on top of the resistive load present at the low MgATP concentrations without Pᵢ.

Effects of Inorganic Phosphate on Dwell-time Histograms of Fluorescent ATP—To distinguish different mechanisms for the inhibition of the actin filament gliding velocity by inorganic phosphate at low substrate concentrations, we employed single molecule detection techniques using fluorescently labeled ATP (Cy3-EDA-ATP). First, we examined the possible effects of inorganic phosphate on the life time of Cy3 signals observed when AB buffer with 5 mM Cy3-EDA-
ATP were infused into a flow cell with myosin-decorated actin filaments immobilized on the BSA-coated assay surface. The assay is illustrated in Fig. 3A. After infusion of the Cy3-EDA-ATP, discrete spots of Cy3 fluorescence appeared and disappeared randomly within the field of view. As proposed by Oiwa et al. (29), we interpreted the appearance of discrete spots as the binding of Cy3-EDA-ATP to immobilized myosin molecules. After cleavage of Cy3-EDA-ATP by myosin II, Pi dissociates from the active site (cf. Scheme 1). This is followed by Cy3-EDA-ADP dissociation, and the spots of Cy3 fluorescence are expected to disappear with the release of Cy3-EDA-ADP. The time during which a spot of Cy3-fluorescence is visible has been termed "dwell time," and the dwell time was assumed to correspond with the period during which the fluorescently labeled nucleotide remains bound to myosin as Cy3-EDA-ATP or Cy3-EDA-ADP (AM-ATP to AM-ADP in Scheme 1 (29)). We included only those signals of Cy3 fluorescence into our analysis that co-localized with an Alexa-488-phalloidin-labeled actin filament. In addition, data recording was limited to 3–4 min to minimize possible effects from changes in substrate and product concentrations.

Fig. 3B shows an example of the intensity time course of a fluorescent spot. The maximum concentration of Cy3-EDA-ATP in the assay buffer was limited to 5 nM. At 5 nM Cy3-EDA-ATP it is highly unlikely that both myosin heads have Cy3-EDA-ATP or Cy3-EDA-ADP bound at the same time. Thus, simultaneous dissociation of both heads from actin is highly unlikely. As a consequence, even if a myosin head in the M-Cy3-EDA-ATP- or M-Cy3-EDA-ADP-P state detached from actin,
it could readily reattach to the actin filament, which is kept in close proximity by the non-dissociated nucleotide-free second head of the myosin molecule.

Fig. 4 shows typical dwell-time histograms of fluorescent signals observed in single assay chambers with 5 nM Cy3-EDA-ATP, both in the absence (Fig. 4A) and in the presence (Fig. 4B) of 5 mM inorganic phosphate. The solid lines are the best possible fits of single exponential functions. The numbers next to each histogram are time constants and errors of the fits to the individual histograms. In several assay chambers the average time constant of the dwell-time distribution for 5 nM Cy3-EDA-ATP alone was found to be 0.81 ± 0.21 s (mean ± S.D.; n = 9 assay chambers) and 0.85 ± 0.28 s (n = 8 assay chambers) for 5 nM Cy3-EDA-ATP plus 5 mM inorganic phosphate; i.e. the dwell-time distribution in the presence of 5 mM P_i was essentially the same as for Cy3-EDA-ATP alone. A small, statistically insignificant increase in dwell time of Cy3 fluorescence, however, cannot be excluded. From the previously proposed concept for reduction in isometric force by inorganic phosphate or for the reduction in the number of driving myosin heads in the gliding assays, one may have expected a significant increase in dwell time as it was expected that after rebinding of inorganic phosphate to the AM-ADP state with formation of a weak binding AM-ADP-P_i intermediate, phosphate first has to be released again before ADP (Cy3-ADP) can finally dissociate from the active site.

To test whether dwell times become more clearly extended in the presence of phosphate analogues with higher affinity for the AM-ADP state, we added 5 mM AlF_4^- as such a phosphate analog (38, 39). Fig. 4C shows a representative dwell-time histogram of fluorescent signals observed in a single assay chamber for 5 nM Cy3-EDA-ATP in the presence of 5 mM AlF_4^- in a set of 9 assay chambers we found the averaged time constants to be 1.34 ± 0.43 s for 5 nM Cy3-EDA-ATP plus 5 mM AlF_4^- versus the 0.81 ± 0.21 s for 5 nM Cy3-EDA-ATP alone. This statistically significant increase in dwell times indicates that AlF_4^- can bind to the AM-ADP intermediate and delays the release of ADP from the active site. The lack of extension of the dwell time by inorganic phosphate, therefore, implies a low probability of rebinding of P_i to the AM-ADP state under our experimental conditions, whereas release of ADP before release of P_i from the AM-ADP-P_i intermediate formed by binding of P_i to the AM-ADP state appears less likely.

Effects of Inorganic Phosphate on the Number of Cy3 Signals per Field of View—To further elucidate the mechanisms by which P_i inhibits filament-gliding velocity at low MgATP concentrations, we established a novel assay in which the number of fluorescent signals in a defined field of view is analyzed (Fig. 5). The number of fluorescent signals per field of view depends on (i) the dwell time of the fluorescently labeled nucleotide, (ii) the number of active sites in the field of view available for binding of Cy3-EDA-ATP, and (iii) the probability of a Cy3-EDA-ATP molecule to bind to the active site of myosin.

If the main effect of phosphate is its binding to M-ADP or AM-ADP states, forming M-ADP-P_i or AM-ADP-P_i intermediates from which it has to be released again before ADP can dissociate, it is expected that the number of signals...
Phosphate Binding to the Active Site of Myosin

Different from this prediction, however, we found that the number of Cy3 signals per field of view seen with 5 nM Cy3-EDA-ATP alone (Fig. 5A, right panel) became much reduced when 5 mM phosphate were present in addition to the 5 nM Cy3-EDA-ATP (Fig. 5A, left panel). Although the reduction in the number of signals per field of view upon the addition of 5 mM phosphate is unexpected from the dwell-time measurements, this reduction instead points to the possibility that the number of active sites available for binding of Cy3-EDA-ATP is reduced, e.g., by binding of phosphate to the empty active site, thus blocking Cy3-EDA-ATP from binding. Fig. 5B shows the number of Cy3 signals in the presence of different Pi concentrations. Note that in the presence of 1–2 mM phosphate the number of fluorescent signals is reduced to 50% of the number observed in the absence of Pi; i.e., in the presence of 1–2 mM Pi, about half of the active sites appear blocked for binding of Cy3-EDA-ATP.

Control for Contaminant Pi—Previous studies have shown that phosphate buffer may contain up to 0.025% contaminant pyrophosphate (40). To rule out that the effects observed upon the addition of 5 mM Pi were due to contaminant Pi, forming a strong binding intermediate of the myosin head (41), we had autoclaved all of our phosphate stock solutions as described by Gyimesi et al. (40) to reduce Mg-PPi by at least 10-fold. In addition, we also examined the effect of Mg-PPi on the number of fluorescent signals per field of view. We applied 100 nM Mg-PPi together with 5 nM Cy3-EDA-ATP with and without 5 mM Pi to flow cells containing myosin-decorated actin filaments. We could not observe any statistically significant change in the number of signals neither when Mg-PPi was added to 5 nM Cy3-EDA-ATP alone nor when added to 5 nM Cy3-EDA-ATP plus 5 mM Pi (Fig. 5C). The addition of 1 µM PPi, in the presence of 5 nM Cy3-EDA-ATP also had no effect (data not shown). Thus, binding of Cy3-EDA-ATP to myosin was unaffected by PPi, at least over the concentration range we have tested.

Control for Photobleaching of Cy3-EDA-ATP—To exclude significant contributions from photobleaching of Cy3-fluorophores to our results, we estimated the bleaching rate of Cy3-EDA-ATP. Because photobleaching can be much affected by the specific local environment of the fluorophore, we aimed at using an approach that resembles our Cy3-EDA-ATP dwell-time measurements as closely as possible, i.e., with Cy3-EDA-ATP bound to myosin. We made use of the observation that with “aged” myosin we still could observe binding of Cy3-EDA-ATP; however, with much extended dwell times. To generate aged myosin we kept myosin extracts at 5 °C for at least 24 h. After this time the aged myosin molecules were applied to flow cells for the aged myosin the observed dwell times of the fluorescent spots upon addition of Cy3-EDA-ATP were extended to 78.25 ± 14.9 s (mean ± S.E., n = 4) at the laser intensity used in our experiments. For different laser intensities we found the time constant of the dwell-time distributions inversely dependent on laser intensity. Thus, we attributed the disappearance of fluorescent signals in assays with aged myosin to photobleaching of the fluorophores rather than dissociation of Cy3-EDA-ATP or Cy3-EDA-ADP. We cannot rule out that the aged myosin may still be capable of proceeding through its ATPase cycle but much more slowly. This control nevertheless shows that

in a fixed field of view will increase with the increase in dwell time as long as the total number of myosin heads and, thus, the number of active sites in the field of view remains unchanged. Because dwell-time distribution with Cy3-EDA-ATP did not change significantly with Pi (Fig. 4), we expected an essentially unchanged number of Cy3 signals with and without phosphate.

![Image](image-url)

**FIGURE 5. Number of fluorescent signals per field of view recorded from assay chambers containing myosin-decorated actin filaments.** A, images for 5 nM Cy3-EDA-ATP plus 5 mM Pi (left panel) and Cy3-EDA-ATP alone (right panel). Each image was generated by accumulation of 10 consecutive frames. Density of myosin molecules was kept comparable for both conditions by collecting data from same field of view. B, summarized data obtained in the absence of Pi, versus the presence of different concentrations of Pi. The concentration of Cy3-EDA-ATP was 5 nM. Data are from two assay chambers of three myosin extracts. In each chamber all six conditions were recorded from the same field of view. Error bars are ± S.D. Note that in presence of 1–2 mM Pi, the number of fluorescent signals is 50% that of the number in the absence of Pi. C, effect of 100 mM pyrophosphate on the number of fluorescent signals in the presence of 5 nM Cy3-EDA-ATP without/with 5 mM Pi. Data are from three assay chambers from three myosin extracts (n = 3). To compare data from different myosin extracts, i.e., different myosin densities in field of view, the number of fluorescent signals per field of view seen with 5 nM Cy3-EDA-ATP alone was set to 1.0 (normalized number of signals). Error bars are ± S.D; ionic strength, 55 mM; temperature, 22–25 °C.
photobleaching (or blinking) of the Cy3-labeled nucleotide is about 100-fold less likely (slower) than the disappearance of the fluorescent signal in our assays with native myosin by dissociation as Cy3-EDA-ADP after cleavage of the Cy3-EDA-ATP and dissociation of Pi, from the active site. Thus, a possible error in the dwell-time histograms of the Cy3-EDA-ATP signals due to photobleaching or blinking is well within the error of the dwell-time measurements.

**DISCUSSION**

In this study we tested the different, previously proposed mechanisms for the inhibition of shortening velocity of muscle fibers and of actin filament gliding velocity in *in vitro* motility assays by inorganic phosphate at low ATP concentrations.

Two assumptions form the basis of the proposal of Hooft *et al.* (26). (i) The load that resists filament sliding is unaffected by inorganic phosphate. Instead, (ii) the population of cross-bridges that “drive” filament sliding, *e.g.* cross-bridges in the AM·ADP state, is reduced by rebinding of phosphate to the AM·ADP state, shifting cross-bridges back to “non-driving”, weak binding states (AM·ADP·Pi, or M·ADP·Pi), just as proposed for the reduction in isometric force by phosphate (16–18). Three lines of evidence argue against this concept. First, we could not see an increased dwell time of fluorescent signals with Cy3-EDA-ATP in the presence of inorganic phosphate under conditions where filament gliding velocity was much reduced (cf. Fig. 4, A and B), *i.e.* where the formation of AM·ADP·Pi or M·ADP·Pi states should have been significant. Comparison with AlF4− showed that rebinding of inorganic phosphate or phosphate analogs, if it occurs, does indeed extend the observed dwell time of fluorescently labeled nucleotide (cf. Fig. 4C). This suggests that ADP release is delayed until AlF4− has dissociated from AM·ADP·AlF4− or M·ADP·AlF4− states. Thus, our dwell-time data imply that, contrary to the prediction of the concept of Hooft *et al.* (26), rebinding of inorganic phosphate to the AM·ADP state is very limited even in our assay with stationary actin filaments. Note that in our assay actin filaments are kept stationary by nucleotide-free myosin heads at the nm concentrations of Cy3-EDA-ATP. Thus, in this assay, if anything, actin filaments should have maintained strain on myosin heads and, thus, should have increased the lifetime of the AM·ADP state compared with gliding assays. Second, in the concept of Hooft *et al.* (26), it is not expected that binding of ATP to the nucleotide-free active site of the myosin head domain is affected by phosphate. This is because in this concept Pi, is assumed to bind to an AM·ADP state and not to the nucleotide-free AM·ADP state. Thus, the reduced number of sites available for binding of ATP, implied by the reduced number of fluorescent signals per field of view when phosphate is raised in the presence of low Cy3-EDA-ATP concentrations (cf. Fig. 5, A and B), again argues against the concept of Hooft *et al.* (26). Third, enhanced fragmentation of actin filaments in the gliding assay is only seen when inorganic phosphate is present at low concentrations of ATP (cf. Fig. 2), *i.e.* when filament sliding is effectively reduced by addition of phosphate. This is consistent with increased resistive load against actin filament sliding, *e.g.* by binding of Pi, to the nucleotide-free actomyosin complex, forming a strong binding AM·Pi state that generates a resistive load against actin filament sliding. Alternatively, increased fragmentation of actin filaments might result from reduced stability of actin filaments in the presence of phosphate, however, only at low MgATP concentrations. This possibility appears unlikely as in trapping experiments in the three-head arrangement (42) no evidence for reduced stability of actin filaments was found in the presence of 5 mM Pi, and μM MgATP.4

By the same line of arguments, the essentially unchanged dwell times of Cy3-EDA-ATP in the presence of phosphate and the reduced number of fluorescent events per field of view upon the addition of phosphate at low Cy3-EDA-ATP concentrations are also inconsistent with one of the concepts proposed by Warshaw *et al.* (25). In this concept phosphate was thought to rebind to the AM·ADP intermediate but to a different site from which phosphate is initially released after cleavage of ATP. Still, it was assumed that ADP release will be delayed until Pi, again has dissociated from this other site. Thus, dwell times with Cy3-EDA-ATP again should be extended in the presence of Pi.

Instead, all of our main findings, (i) no effect of Pi, on dwell times of fluorescent signals in the presence of Cy3-EDA-ATP, (ii) a reduced number of Cy3 signals per field of view, and (iii) increased fragmentation of actin filaments, both seen in the presence of Pi, at low ATP concentrations, are fully consistent with binding of inorganic phosphate to the nucleotide-free active site of the myosin head domain. The formed AM·Pi complex blocks ATP from binding to the active site and generates additional impedance against filament sliding.

**Affinity of Phosphate for the Actin-attached Myosin Head—**

Our assay in which we count the number of Cy3 signals per field of view allows determination of the binding constant of inorganic phosphate for its binding site. At 5 nm Cy3-EDA-ATP, the second order rate constant of Cy3-EDA-ATP binding to myosin (1.1 × 106 M−1 s−1 (29)) together with the dwell time of the fluorescent signal of about 1 s yield a ratio of myosin heads with fluorescent nucleotide versus nucleotide-free myosin heads of about 1:200. Thus, at such a low concentration of Cy3-EDA-ATP, the number of Cy3 signals per field of view very closely reflects the number of myosin heads available for binding of Cy3-EDA-ATP provided the total number of myosin heads in the field of view remains unchanged throughout the measurements. This was ensured by using the same field of view for all the different phosphate concentrations and by testing the starting conditions at the very end again to rule out loss of myosin molecules during the course of the measurements. The observed reduction in the number of Cy3 signals to half in the presence of 1–2 mM phosphate, thus, indicates that at these concentrations phosphate blocks half of the nucleotide binding sites of the myosin molecules from binding of Cy3-EDA-ATP. These data yield an equilibrium binding constant for phosphate to its binding site of 0.5–1 mM−1. Note that this is the binding constant of Pi, for actin-attached myosin (AM state) as we used myosin-decorated actin filaments, and actin filaments did not dissociate from the assay surface in the presence of 5 mM Pi.

**The AM·Pi Intermediate Has Strong Actin Binding Properties—**

Reduced gliding velocity and enhanced fragmentation of actin

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4 W. Steffen, Dept. Molecular and Cell Physiology, Hannover Medical School, D-30625 Hannover, Germany, personal communication.
filaments upon formation of the AM-P_i complex imply that forces that resist filament sliding are increased by the AM-P_i complex. This suggests that (i) the AM-P_i complex binds tightly to actin and (ii) that its dissociation from actin is slow, i.e. properties that are characteristic for strong binding intermediates of the myosin head domain (43). This is further supported by the previously reported shift in fiber stiffness versus speed of the stretches applied to fibers for the stiffness measurements (21); in the presence of inorganic phosphate fiber stiffness remained higher than without P_i when velocity of fiber stretch was reduced. This again implies that accumulation of myosin heads in the AM-P_i state results in slow dissociation from actin, i.e. that in the AM-P_i state myosin is strongly bound to actin with slow dissociation (43).

In Dwell-time Assays Binding of Inorganic Phosphate to the AM-ADP State Is Unfavorable—The longer dwell times seen with Cy3-EDA-ATP in the presence of AlF_4 (Figs. 4C) show that dwell times of fluorescent nucleotide can be extended, at least in the presence of P_i analogs. This observation argues against release of Cy3-EDA-ADP before the release of P_i or P_i analogs as the reason for the unchanged dwell times in the presence of P_i. Instead, these data imply that in the motility assays the life time of the AM-ADP state is rather limited, presumably because of lack of strain being exerted on actin-bound myosin heads, even if actin filaments are held stationary by other, nucleotide-free myosin heads at the 5 nM Cy3-EDA-ATP. Because of the short lifetime, rebinding of P_i to the AM-ADP state becomes unlikely, and rebinding to the short-lived AM-ADP state can only be detected with the P_i analog AlF_4, as (i) its affinity for the AM-ADP complex is higher (38), and (ii) extension of dwell times is expected to be much longer as dissociation of AlF_4 from the AM-ADP-AlF_4 complex is much slower compared with dissociation of P_i from the AM-ADP-P_i intermediates (cf. “trapping” of AlF_4 (38, 39)).

Relation to Previous Work—Indications of possible binding of inorganic phosphate to the empty nucleotide binding pocket came from solution studies (22–24), from reduction of actin filament gliding velocity in the in vitro motility assay conditions, our approach of directly probing the fraction of actin filament gliding velocity in motility assays at lower ionic strengths (70–80 mM). These two estimates are still close to those from solution studies and our assay, although dependence of unloaded shortening velocity in muscle fibers and of actin filament gliding velocity in motility assays on the fraction of strongly bound myosin heads and, thus, on the fraction of active sites occupied by P_i is highly non-linear. For motility assay conditions, our approach of directly probing the fraction of myosin heads available for ATP binding by the number of fluorescent spots seen in the presence of Cy3-EDA-ATP (cf. Fig. 5) allows a more direct estimate of the affinity of phosphate for the AM state.

Relevance of P_i Binding to the Empty Nucleotide Binding Site—A large number of single molecule experiments, whether on processive or non-processive myosins, are performed at μm or even nM concentrations of ATP. This is done to e.g. increase event lifetimes in trapping experiments or to slow down processive stepping. Low MgATP concentrations facilitate detection of events and localization of fluorescent signals when step sizes or step size distributions of motor proteins are to be determined. As shown in this study, however, μm or lower MgATP concentrations also facilitate the formation of an AM-P_i complex when phosphate is present. Formation of an AM-P_i complex with high affinity for actin, however, is expected to affect event lifetimes and may even generate additional displacement sub-steps. Thus, binding of P_i to the nucleotide-free active site merits consideration when phosphate is used in single molecule assays to dissect molecular events of force-generation and movement (e.g. Refs. 44–46), an approach that may develop into as central a tool for single molecule assays as it became for fiber studies (e.g. Refs. 4, 8–18, 20, and 21).

Acknowledgments—We thank Dr. Kazuhiro Oiwa, Kobe Advanced ICT Research Center, National Institute of Information and Communications Technology, Kobe, Japan, for generously providing Cy3-EDA-ATP. We also thank David Luckhaus and Ante Rudocaj for support.

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