Calcium Functionally Uncouples the Heads of Myosin VI*

Carl A. Morris‡§, Amber L. Wells‡§, Zhaohui Yang, Li-Qiong Chen, Corradina V. Baldacchino, and H. Lee Sweeney¶

From the ‡Department of Physiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6085

This study examines the steady state activity and in vitro motility of single-headed (S1) and double-headed (HMM) myosin VI constructs within the context of two putative modes of regulation. Phosphorylation of threonine 406 does not alter either the rate of actin filament sliding or the maximal actin-activated ATPase rate of S1 or HMM constructs. Thus, we do not observe any regulation of myosin VI by phosphorylation within the motor domain. Interestingly, in the absence of calcium, the myosin VI HMM construct moves in an in vitro motility assay at a velocity that is twice that of S1 constructs, which may be indicative of movement that is not based on a “lever arm” mechanism. Increasing calcium above 10 μM slows both the rate of ADP release from S1 and HMM actomyosin VI and the rates of in vitro motility. Furthermore, high calcium concentrations appear to decouple the two heads of myosin VI. Thus, phosphorylation and calcium are not on/off switches for myosin VI enzymatic activity, although calcium may alter the degree of processive movement for myosin VI-mediated cargo transport. Lastly, calmodulin mutants reveal that the calcium effect is dependent on calcium binding to the N-terminal lobe of calmodulin.

Myosin VI was the first myosin demonstrated to move toward the pointed (−) end of an actin filament (1). This discovery was based on the hypothesis that reverse direction would require a redesign of the domain of myosin that couples changes in the state of the nucleotide binding pocket and actin-myosin interface to movements of the myosin light chain binding domain (the myosin “lever arm”). Indeed, cryoelectron microscopy revealed that upon ADP release, the effective myosin VI lever arm rotates in the opposite direction (i.e. toward the pointed end of the actin filament) compared with other characterized myosins (1).

The light chain-binding region of each head of the myosin VI dimer is thought to consist of a single calmodulin bound to the heavy chain “IQ” motif. This light chain binding region is generally believed to act as a “lever arm” that amplifies nucleotide state-dependent structural changes within the core of the myosin motor domain (for review, see Refs. 2 and 3). For both myosin II and myosin V, the length of the “lever arm” has been shown to correspond to the step size associated with a single ATPase cycle (4, 5). Recent kinetic and single molecule data demonstrate that myosin VI has a high duty ratio (i.e. remains strongly bound to actin for >90% of its actomyosin ATPase cycle) and is processive (6–8). Cryoelectron microscopy studies of myosin VI bound to actin showed a very compact effective “lever arm” that appears too small to account for the large step size of myosin VI (1, 7). Furthermore, the single molecule data reveal that myosin VI has a broad distribution of step sizes centered on 30 nm (7), which also is not consistent with a lever arm mechanism. Thus, it is unclear what role the region containing the IQ motif of myosin VI is playing in motility. To provide further insight into the mechanism of movement, we examined the motility of myosin VI S1 and HMM constructs. The expectation based on myosin V is that for a lever arm mechanism, the asymmetry of the lever arm swing (9) will allow an ensemble of the S1 constructs to move actin filaments at a high percentage of the velocity of the HMM construct.

Whereas the light chain-binding region of myosin VI may not function as a conventional lever arm, the fact that it contains calmodulin could allow some form of regulation by calcium. As shown for myosin V, there could be altered motor function of myosin VI as a function of calcium concentration (10, 11). A recent report suggested that calcium binding to myosin VI serves as an “on/off” switch for myosin VI motility (12). Herein, we perform a more detailed investigation of the nature of calcium regulation of myosin VI using S1 and HMM myosin VI constructs.

Myosin VI, like a number of class I myosins, has a putative phosphorylation site in one of its actin binding loops (the “HCM” loop) (13). There is evidence that Thr406 is a PAK phosphorylation site (12, 14), and recently Yoshimura et al. (12) raised the possibility that phosphorylation of the loop serves as an “on/off” switch for motility. However, this mechanism is inconsistent with a recent kinetic study of myosin VI using mutants T406E and T406A, designed to mimic the phosphorylated and dephosphorylated loop, respectively (6). Those results suggested that phosphorylation does not alter the rate-limiting step in the actomyosin VI ATPase cycle (ADP release); rather, phosphorylation increases the rate of phosphate release, thereby increasing the duty cycle. Since ADP release also limits in vitro motility, the expectation, based on the work of De La Cruz et al. (6), was that phosphorylation would not be an on/off switch for either ATPase activity or motility, contrary to the conclusions of Yoshimura et al. (12). To resolve the apparent contradictions pertaining to the mechanism and regulation of movement of myosin VI on actin filaments, we examined the in vitro motility and solution kinetics of both single- and double-headed myosin VI with different levels of phosphorylation and at different calcium concen-
trations. Additionally, we examined the site of calcium action using calmodulin mutants with either N- or C-terminal calcium binding eliminated.

EXPERIMENTAL PROCEDURES

Myosin VI Expression and Purification—To create single-headed, S1-like myosin VI constructs (S1), porcine myosin VI wild-type cdNA (15) was truncated at Gly260. A FLAG tag (encoding GYKDKKDDDE) was inserted at the C terminus to facilitate purification (16), and a Myc tag (encoding EQLISEEDL) was inserted preceding the FLAG tag for use in vitro motility assays (17). The recombinant heavy chain protein contains the motor domain and the single calmodulin/light chain-binding site (IQ motif). The double-headed, HM-like construct was truncated at Arg264 to include 20 native heptad repeats of predicted coiled-coil with a C-terminal leucine zipper (GCN4) to ensure dimerization and improve protein yield (18) followed by the Myc and FLAG tag sequences. For comparative motility studies, we used two myosin V constructs, single-headed, 1-IQ (S1-like) and double-headed, 6-IQ (HMM) proteins. The double-headed construct was produced as previously described (7). Construction of the single-headed construct involved truncation of the myosin V heavy chain at Lys267. As for myosin VI, the FLAG and Myc epitope were added for purification and motility, respectively. Expression of the recombinant single- and double-headed fragments of myosin VI was accomplished via infection of SF9 insect cells with a viral expression vector (baculovirus) capable of driving high level expression of foreign proteins. The SF9 cells were co-infected with recombinant baculovirus using the myosin V heavy chain and recombinant baculovirus for calmodulin expression. Details of the protein expression and purification have been published (1, 19). Greater than five separate protein preparations were used in the experiments described in this study to account for any preparation-specific effects.

Calmodulin Mutants—To generate calmodulin with either the N- or C-terminal calcium binding sites eliminated, the cdNA of chicken calmodulin was mutated to make E104A,E140A for the C-terminal mutant and E31A,E67A for the N-terminal mutant. The mutant cdNA constructs were then inserted into a viral expression vector for co-transfection with the myosin VI constructs or into a bacteria expression vector to generate exogenous calmodulin for use during the kinetic and motility experiments.

Protein and Reagents—mantADP2 was synthesized as previously described (20). Actin was prepared from acetone powder as described (21) and gel-filtered. Phallolidin (Sigma) was added to stabilize the F-actin following polymerization and dialysis into the appropriate buffer. Appyrase at 0.02 units/ml (Sigma) was used to remove contaminating nucleotides. Purified PAK3 protein was purified on SDS-polyacrylamide gels to determine purity of the preparation, and the protein concentrations were measured colorimetrically using the Bio-Rad protein assay.

The solutions used in both the kinetic measurements and the in vitro motility assay were at an ionic strength of 78 mM and contained 0.37 g/l SDS-polyacrylamide gels to determine purity of the preparation, and the protein concentrations were measured colorimetrically using the Bio-Rad protein assay. The solutions used in both the kinetic measurements and the in vitro motility assay were at an ionic strength of 78 mM and contained 0.37 g/l SDS-polyacrylamide gels to determine purity of the preparation, and the protein concentrations were measured colorimetrically using the Bio-Rad protein assay. The solutions used in both the transient kinetic measurements and the in vitro motility assay were at a protein concentration of 1 mg/ml F-actin. NaCl, 40 mM ATP, 10 mM MgCl2, 1 mM DTT, and 0.1% methylcellulose were added to limit further ATP hydrolysis and protein aggregation. All solutions were preincubated with the appropriate reagents prior to 1:1 mixing for measurement. mantADP was directly excited at 365 nm, and the fluorescence was measured using a 400-nm long pass filter. N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)-coumarin-3-carboxamide (MDCC)-labeled phosphate-binding protein (22) was excited at 425 nm, and the fluorescence was monitored using a 455-nm colored glass filter.

The ATPase activity of myosin VI-mantADP complex was mixed 1:1 with 4 mM Mg-ADP. A single exponential was used to fit the time course of the fluorescence decrease. The experiments were performed at 30 °C.

Transient P Release—Phosphate release was measured using the stopped-flow apparatus in sequential mix mode. Myosin VI S1 (4 μM; starting concentration) was mixed with Mg-ATP (400 μM), aged for 300 ms to populate the actomyosin-ADP-P state, and then mixed with F-actin. The initial burst was fit by a single exponential with a slope. 2 mM ADP was added to the F-actin solution to limit further ATP binding. N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)-coumarin-3-carboxamide (MDCC)-labeled phosphate binding protein (PBP) was added to the initial myosin and actin solutions to produce a final PBP concentration of 4 μM. All solutions and the stopped-flow were preincubated with a Pm mosaic solution containing 0.5 units/ml purine nucleoside phosphorylase and 250 μM 7-methylguanosine. The experiments were performed at 25 °C.

Steady State ATPase—The Mg-ATPase activity of the myosin constructs was determined using the NADH-coupled assay in the stopped-flow or spectrophotometer by monitoring the absorbance change at 340 nm. The ATPase solution was composed of the described pCa solutions with 200 μM NADH (Sigma), 250 μM phosphoenol pyruvate (Sigma), 10 mM MgCl2, 1 mM NaF, 10 mM Na2ATP, 20 nM rhodamine-phalloidin-labeled F-actin was added to the MgCl2 washes out, and 1 mg/ml bovine serum albumin was added to block non-specific sites. The myosin VI was incubated for 2 min, washed out, and 1 mg/ml bovine serum albumin was added to block nonspecific sites. The myosin VI was left to incubate for 2 min, followed by washing with solution containing bovine serum albumin. This was followed by the addition of 5 μM unlabelled F-actin to block any nonspecific myosin molecule interactions (23). The activating solution was composed of the described pCa solutions supplemented with 0.3–0.5% methcylcellulose and 2.5 mg/ml glucose, 0.1 mg/ml glucose oxidase, 0.02 mg/ml catalase, and 2 mM dithiothreitol to inhibit photobleaching. The single- and double-headed myosin VI constructs were attached to the nitrocellulose-coated glass coverslips via an antibody directed against the C-terminal Myc tag (23). The Myc antibodies were added to the flow cell, incubated for 2 min, and washed out, and 1 mg/ml bovine serum albumin was added to block nonspecific sites. The myosin VI was added to the flow cell, and the sliding speed of the actin filaments was quantified using a fluorescence microscope equipped with a low light level camera. The raw data were recorded onto videotape and analyzed offline using a motor and feedback system (Santa Rosa, CA), as described previously (23).

RESULTS

Phosphorylation State of Expressed Wild-type Myosin VI—In Fig. 1A, the untreated, wild-type myosin VI S1 following expression and purification from SF9 cells is shown with myosin VI treated with either PAK3 or λ-phosphatase, probed on a Western blot with an anti-phosphoepitope antibody. The untreated myosin VI samples show relatively high levels of three-
Myosin VI Regulation

FIG. 1. A, Western blot of S1 wild-type and T406E myosin VI before (Untreated) and after either λ-phosphatase (+λ) or PAK3 (+PAK) treatment. The myosin VI was incubated with λ-phosphatase for 30 min at 30 °C in a buffer containing 2 mM MnCl2 (see Experimental Procedures) or with PAK3 at 37 °C in the presence of 1 mM Mg2+ATP. The level of myosin VI phosphorylation was then analyzed by SDS-PAGE and Western blot using an anti-phosphoserine antibody (Zymed Laboratories Inc.) to detect phosphorylation of different myosin VI preparations. Control experiments using an anti-phosphorepine antibody (Zymed Laboratories Inc.) showed no change in the level of phosphorylation following treatment with either PAK3 or λ-phosphatase (data not shown).

Actin-activated ATPase activity of myosin VI with or without λ-phosphatase treatment. The ATPase activity was determined at 30 °C in a pCa 9.0 buffer containing 10 mM imidazole (pH 7.3), 46 mM KCl, 2.4 mM MgCl2, 2 mM total EGTA, and 2 mM dithiothreitol with 2 mM Mg2+ATP as described under “Experimental Procedures.” Two separate protein preparations were used for both the S1 and HMM. A, the ATPase activity of myosin VI S1 with (open circles) or without λ-phosphatase treatment (filled circles). B, the ATPase activity of untreated myosin VI HMM (filled circles) or following λ-phosphatase treatment (open circles). In both graphs, the solid lines through the data represent the best fit of the equation $y = \left( V_{\text{max}} + [\text{actin}] \right)/K_{\text{ATPase}} + [\text{actin}]$. For the S1, the $V_{\text{max}}$ was $6.2 \pm 0.2$ head$^{-1}$ s$^{-1}$ (mean ± S.E.) untreated versus $6.2 \pm 0.3$ head$^{-1}$ s$^{-1}$ treated, and the $K_{\text{ATPase}}$ was $6.3 \pm 0.7$ μM treated versus $5.9 \pm 0.5$ μM untreated. For the HMM, the $V_{\text{max}}$ was $3.2 \pm 0.2$ head$^{-1}$ s$^{-1}$ untreated versus $3.5 \pm 0.2$ head$^{-1}$ s$^{-1}$ treated, and the $K_{\text{ATPase}}$ was $1.8 \pm 0.3$ μM treated versus $1.8 \pm 0.3$ μM untreated.

FIG. 2. Actin-activated ATPase activity of myosin VI with or without λ-phosphatase treatment. The ATPase activity was determined at 30 °C in a buffer containing 10 mM imidazole (pH 7.3), 46 mM KCl, 2.4 mM MgCl2, 2 mM total EGTA, and 2 mM dithiothreitol with 2 mM Mg2+ATP as described under “Experimental Procedures.” Two separate protein preparations were used for both the S1 and HMM. A, the ATPase activity of myosin VI S1 with (open circles) or without λ-phosphatase treatment (filled circles). B, the ATPase activity of untreated myosin VI HMM (filled circles) or following λ-phosphatase treatment (open circles). In both graphs, the solid lines through the data represent the best fit of the equation $y = \left( V_{\text{max}} + [\text{actin}] \right)/K_{\text{ATPase}} + [\text{actin}]$. For the S1, the $V_{\text{max}}$ was $6.2 \pm 0.2$ head$^{-1}$ s$^{-1}$ (mean ± S.E.) untreated versus $6.2 \pm 0.3$ head$^{-1}$ s$^{-1}$ treated, and the $K_{\text{ATPase}}$ was $6.3 \pm 0.7$ μM treated versus $5.9 \pm 0.5$ μM untreated. For the HMM, the $V_{\text{max}}$ was $3.2 \pm 0.2$ head$^{-1}$ s$^{-1}$ untreated versus $3.5 \pm 0.2$ head$^{-1}$ s$^{-1}$ treated, and the $K_{\text{ATPase}}$ was $1.8 \pm 0.3$ μM treated versus $1.8 \pm 0.3$ μM untreated.
control steady state measurements indicated that the phosphorylation state did not significantly change the calcium dependent effect observed when the calcium concentration was elevated from pCa 9.0 to pCa 4.0 (data not shown).

Control experiments were performed to determine whether excess calmodulin was necessary to maintain maximal activity of the myosin VI constructs. For these experiments, 0–30 μM wild-type chicken calmodulin, expressed in *Escherichia coli* and purified, was mixed with myosin VI S1. The actin-activated ATPase activity was measured in a pCa 4.0 or pCa 9.0 solution at 30 °C using the NADH-coupled assay, as described in the legend to Fig. 2 and under “Experimental Procedures,” in the presence of 20 μM F-actin. At both pCa 4.0 and pCa 9.0, the ATPase rates were similar regardless of the calmodulin concentration, in agreement with the results of Yoshimura et al. (12). The activity ranged from 4.2 to 4.6 s^{-1} at pCa 9.0 and from 3.0 to 3.3 s^{-1} at pCa 4.0 (data not shown). Further, as shown previously by Yoshimura et al. (12), control steady state measurements indicated that the phosphorylation state did not significantly change the calcium dependent effect observed when the calcium concentration was elevated from pCa 9.0 to pCa 4.0 (data not shown).

### Table I

**Comparison of in vitro motility of wild type myosin V and VI constructs**

| Actin filament velocity | S1, single-headed | HMM, double-headed |
|-------------------------|------------------|-------------------|
| Myosin V                | 322 ± 32         | 459 ± 45          |
| Myosin VI (untreated)   | 131 ± 20         | 307 ± 39          |
| Control (for λ-phosphatase) | 238 ± 64   | 222 ± 65          |
| Treated (+ λ-phosphatase) |                 |                   |

---

**In Vitro Motility as a Function of Calcium Concentration—**

The results of in vitro motility assays on single- and double-headed myosin VI over the calcium range pCa 9.0 to pCa 3.0 are shown in Fig. 4. In the absence of calcium, the HMM species moves actin filaments at slightly greater than twice the speed of the S1 constructs (307 versus 131 nms^{-1}). This is a much greater difference than was seen in the case of single-headed versus double-headed myosin V (Table I). At high calcium concentrations, the speed of both constructs is reduced, but to a much greater extent for the HMM construct. Increasing the calcium concentration reduced the single-headed myosin VI motility from 131 to 80 nms^{-1}, a reduction to 60% from pCa 9.0 speeds, whereas the myosin VI HMM motility was reduced to 30% of the pCa 9.0 motility, dropping from a speed of 307 to 103 nms^{-1}. The greatest reduction in sliding speed is observed between pCa 6.0 and pCa 5.0 for the HMM construct, thus over a physiological calcium concentration range. At high calcium concentrations, the motility is similar for the single- and double-headed constructs.

**Table II**

**Effect of [Ca^{2+}] on myosin VI actin-activated ATPase activity**

The experiments were performed at 30 °C using the NADH-coupled assay system as described under “Experimental Procedures.” The final [Mg^{2+}·ATP] was 2 mM with 0.37 mM free Mg^{2+}. The kinetic parameters were determined by fitting the data to a hyperbola of the form \( v = V_{\text{max}} \left[\text{actin} \right] / \left( K_{\text{ATPase}} + \left[\text{actin} \right] \right) \). The data shown are mean ± S.E. of the fits using four separate protein preparations.

| pCa 9.0 | pCa 4.0 |
|---------|---------|
| **K_{\text{ATPase}} [μM]** | **V_{\text{max}} [nms^{-1}]** | **K_{\text{ATPase}} [μM]** | **V_{\text{max}} [nms^{-1}]** |
| S1 (single-headed) | 5.7 ± 0.7 | 5.3 ± 0.2 | 4.4 ± 0.3 | 3.7 ± 0.1 |
| HMM (double-headed) | 1.2 ± 0.2 | 2.8 ± 0.2 | 1.1 ± 0.2 | 3.2 ± 0.2 |

---

**Discussion**

Myosin VI Phosphorylation at Thr^{606}—We have shown that myosin VI, in our hands, is expressed and purified under conditions that maintain the myosin in a highly phosphorylated state (Fig. 1). Assuming that PAK3 phosphorylates nearly 100% of the myosin, we find ~80–90% of both myosin VI heads are phosphorylated following purification. Blot analysis and densitometry of the T406E mutant suggests that other threonines within our constructs are not phosphorylated by PAK3 or...
The actin-activated ATPase activity (per head) of myosin VI S1 (closed circles) and HMM (open circles) as a function of calcium concentration. The ATPase activity was measured at 30 °C using the NADH-coupled assay as described in the legend to Fig. 2 and under "Experimental Procedures." The pCa was varied by altering the relative amount of Ca^2+--K^-EGTA and K^-EGTA or, for pCa 3.0 and 4.0, directly adding CaCl_2. Comparison of the ATPase activity of the myosin VI S1 at high and low [Ca^2+] indicated a significant reduction in activity at calcium concentrations of pCa 5.0 and higher (p < 0.05). The data are shown as mean ± S.D. for three or four separate measurements and preparations.

![Figure 3](image_url)

FIG. 3. The actin-activated ATPase activity (per head) of myosin VI S1 (closed circles) and HMM (open circles) as a function of calcium concentration. The ATPase activity was measured at 30 °C using the NADH-coupled assay as described in the legend to Fig. 2 and under "Experimental Procedures." The pCa was varied by altering the relative amount of Ca^2+--K^-EGTA and K^-EGTA or, for pCa 3.0 and 4.0, directly adding CaCl_2. Comparison of the ATPase activity of the myosin VI S1 at high and low [Ca^2+] indicated a significant reduction in activity at calcium concentrations of pCa 5.0 and higher (p < 0.05). The data are shown as mean ± S.D. for three or four separate measurements and preparations.

![Figure 4](image_url)

FIG. 4. In vitro motility of myosin VI. The rate of actin filament sliding by the myosin VI S1 (closed circles) or HMM (open circles) construct is shown as a function of free Ca^2+ concentration. Motility was observed in pCa buffers composed of 10 mM imidazole (pH 7.3), 46 mM KCl, 2.4 mM MgCl_2, 2 mM total EGTA, 0.4% methylcellulose, 2.5 mg/ml glucose, 0.1 mg/ml glucose oxidase, 0.02 mg/ml catalase, 2 mM dithiothreitol, and 2 mM Mg^2+-ATP. Calcium concentrations were adjusted as described in the legend to Fig. 4 and under "Experimental Procedures." Actin filament sliding speed is shown as mean ± S.D. for 218–297 filaments. The addition of excess calmodulin is unnecessary, since Ca^2+ does not cause the CaM to dissociate, as first reported by Yoshimura et al. (12) and confirmed by stoichiometry on gels (data not shown).

![Figure 5](image_url)

FIG. 5. Site of calcium dependence of the maximal actin-activated ATPase of myosin VI S1. The steady state ATPase activity of myosin VI following coexpression with the N- and C-terminal calmodulin mutants was determined at 0–30 μM actin in the presence (pCa 4.0) and absence (pCa 9.0) of calcium. The experiments were performed at 30 °C using the NADH-coupled assay as described in the legend to Fig. 2 and under "Experimental Procedures." The calmodulin mutants were coexpressed with the myosin VI S1, and a 6 μM excess of the calmodulin mutant was added to the experimental solution. The V_{max} steady state ATPase activity is shown as means ± S.E. of the fit. Calcium dependence of the ATPase activity was observed for the C-terminal mutant (E104A, E140A), but the N-terminal mutant (E31A, E67A) showed no such dependence.

| pCa 9.0 | pCa 4.0 |
|---------|---------|
| MV (S1, single-headed) | 5.2 ± 1.1 | 3.7 ± 0.8 |
| MV (HMM, double-headed) | 5.4 ± 0.4 | 3.6 ± 0.5 |

TABLE III

Effect of [Ca^{2+}] on mantADP release rate of wild type myosin VI

All experiments were performed at 30 °C using the pCa 4.0 or pCa 9.0 buffers described under "Experimental Procedures." Either 1 μM (S1) or 0.5 μM (HMM) myosin VI was incubated in the pCa solution with 50 μM mantADP and 5 μM F-actin. The myosin VI was rapidly mixed with a similar pCa solution containing 4 mM MgADP (2 μM final concentration). The time course of the fluorescence decrease was fit with a single exponential. The data are shown as mean ± S.D. taken from 5–7 separate traces averaged from two experiments using two separate protein preparations.

dephosphorylated by λ-phosphatase (Fig. 1). Further controls using an anti-phosphoserine antibody show no changes in phosphorylation following PAK3 or λ-phosphatase treatment (data not shown).

The data demonstrate that the kinetic parameters of the actin-activated ATPase for the single- or double-headed wild-type myosin VI are unchanged whether or not Thr^{406} is phosphorylated (Fig. 2). The kinetics in either case are intermediate to the values published for the T406E and T406A S1 mutants (6), which differed primarily in the K_{ATPase} and the actin-activated rate of P_i release. The lack of a change in the K_{ATPase} as a function of phosphorylation suggests that the rate of P_i release from actomyosin VI is unchanged. Indeed, in the present experiments, a comparison of the P_i release rate at 40 μM actin revealed no effect of phosphorylation. The rate measured in this study for the wild-type protein with or without phosphorylation of Thr^{406} (30–30 s^{-1}) was intermediate to the values measured for the T406E and T406A mutants (6). Thus, the mutants are not perfect mimics of the phosphorylated and dephosphorylated states. Whereas it was originally suggested (6), based on analysis of the mutants, that phosphorylation of Thr^{406} results in an increased duty ratio, thereby modulating the degree of processivity, we now can find no effect of authentic Thr^{406} phosphorylation.

As predicted from the solution kinetics, in vitro motility is not modulated by phosphorylation of Thr^{406} (Table I); nor is it altered by the Thr^{406} mutations (data not shown). This is not at odds with the ATPase data of Yoshimura et al. (12), although our motility data differs from theirs. They concluded that although phosphorylation did not affect the actin-activated ATPase activity, it served as an on/off switch for motility as demonstrated by a reduction in the quality of motility (percentage of filaments moving) upon dephosphorylation of myosin VI. Their ATPase data are in general agreement with ours but are inconsistent with their own motility results in that no kinetic parameters are changing that affect either the V_{max} or K_{ATPase}.

Single- Versus Double-headed Myosin VI Function—It is interesting to note that the velocity of actin filament sliding of the myosin VI HMM construct in the absence of calcium (pCa 9.0) is more than twice that of the S1 construct (307 versus 131
Myosin VI Regulation

nm s⁻¹). For myosin V (Table I), the HMM moves at a speed that is only ~40% greater than that of the S1 (459 versus 322 nm s⁻¹). The difference observed in myosin V probably is a manifestation of the asymmetry of the lever arm positions in the pre- and post-power stroke states, as recently discussed (9). This asymmetry gives rise to a step size (stroke) of ~25 nm for the myosin V-6IQ S1 and a step size of ~36 nm for the HMM molecule (9, 24). Further, a shortened myosin V construct with four light chain binding motifs (4-IQ) exhibited reduced step sizes of ~16 and ~24 nm for the S1 and HMM constructs, respectively (5). Thus, it has been suggested that the light chain binding, or neck, region of myosin V functions as a semirigid lever arm (5, 9, 24, 25), similar to the neck region of myosin II (4). The extended lever arm acts to amplify small nucleotide-induced conformational changes within the head of the myosin to generate directed motion of the molecule itself and any cargo bound to the tail region. If a similar mechanism was responsible for the difference observed between the single- and double-headed myosin VI, the actin filament sliding speed would be reduced from 307 nm s⁻¹ for HMM to ~190 nm s⁻¹ for the S1. The observation that the ratio of single- and double-headed motility speeds is greater than predicted for myosin VI could be related to the fact that myosin VI probably does not move via a conventional lever arm mechanism (7).

Alternatively, the myosin VI HMM construct could have accelerated ADP release (rate-limiting for motility) compared with the S1. Thus, we measured mantADP release from the single- and double-headed actomyosin VI complexes (Table III). All of these results are consistent with the rate of ADP release from single- or double-headed actomyosin VI being the same and limiting both in vitro motility and actin-activated ATPase assays. At pCa 9.0, the S1 and HMM mantADP release rates were similar at 5.2 and 5.4 s⁻¹, respectively. For the S1 construct, the rate of mantADP release was in good agreement with the steady state rate of actin-activated ATPase activity in the absence of calcium (5.3 s⁻¹; Table II). However, for myosin VI HMM, the rate of mantADP release at pCa 9.0 (5.4 s⁻¹; Table III) was approximately twice the actin-activated ATPase rate per head (2.8 s⁻¹; Table II), suggesting that the two heads are not functioning independently. Rather, these results are consistent with gating between the heads of myosin VI on actin, as previously reported for the double-headed T406E mutant (6) and described for myosin V (9). By gating, we mean that if one head of the two-headed myosin VI molecule is strongly bound to actin, it blocks the other head from continuing through its ATPase cycle (product release) until the first head detaches from actin.

For our myosin VI HMM constructs, single molecule analyses at 25 °C determined an average step size of 30 nm and an in vitro motility speed of 250 nm s⁻¹ (7). This predicts a rate-limiting step (ADP release) of ~8 s⁻¹. This is in reasonable agreement with the kinetics in this study (given that we are measuring mantADP release) and in the previous work of De La Cruz et al. (6). The motility of the myosin VI S1 construct is slightly less than half that of the HMM. Assuming that the rate of ADP release is unchanged in the motility assay, as appears to be the case for the HMM, the extrapolated step size of the myosin VI S1 would be ~12.5 nm. This is too large a step size (stroke) to be explained by the short “lever arm” seen in the cryoelectron microscopy reconstructions of this construct (1). Thus, even for myosin VI S1, the mechanism of movement is unclear. The effective lever arm region of myosin VI could be a highly flexible region in certain nucleotide states that presents itself as a small, almost globular domain in cryoelectron microscopy images of A.M.ADP or rigor (1). This region may act similarly to the flexible linker in kinesin, which is thought to switch between very different structural forms during the ATPase cycle (26).

Effect of Calcium on Myosin VI Function—Elevating calcium from pCa 9.0 to pCa 4.0 did not have a major effect on either the Vmax or the KATPase of the actin-activated ATPase rate of the double-headed myosin VI (Fig. 3). This is consistent with the observations of Yoshimura et al. (12), although our KATPase values are significantly lower for phosphorylated myosin VI than they reported. Interestingly, the Vmax of the myosin VI S1 ATPase was lowered by calcium.

Examination of the ADP release rate from both the S1 and HMM constructs demonstrated that elevated calcium (pCa 4.0) slowed the release to a similar extent in both cases (Table III). Whereas this explains the observed decrease in the S1 ATPase rate at pCa 4.0, it is puzzling in the context of the HMM, since there is a trend for the ATPase activity to either slightly increase or stay the same as calcium levels are increased. The explanation to this apparent discrepancy is revealed by observing the in vitro motility of the single- and double-headed constructs as a function of calcium. Whereas calcium does not function as an “on/off” switch for myosin VI motility, it does slow the rate of actin filament sliding for both single and double-headed species, consistent with the effect on the ADP dissociation rate. For the myosin VI S1, the slowing is proportional to the slowing of the ADP release rate. For the HMM, the slowing of the in vitro motility is twice as much as would have been predicted from the ADP release rate. A close examination of the data in Fig. 4 reveals that at low calcium concentrations (~1 μM), the myosin VI HMM moves at a velocity that is slightly more than twice as fast as the S1 species. However, as calcium levels are increased, the velocities of the two constructs become nearly the same, with the most significant reduction in speed occurring between [Ca²⁺] of 1 and 10 μM. In essence, it appears that calcium breaks the coordination between the heads so that the two heads of the double-headed construct are acting independently in the motility assay. This also explains the paradoxical observation that there may be a slight increase in the Vmax of the actin-activated ATPase at high calcium, although the rate-limiting step (ADP release) has been decreased. In fact, as discussed above and published recently (6), coordination between the heads allows “gating” in the absence of calcium, so that the Vmax of the myosin VI HMM is approximately half the ADP release rate. At high [Ca²⁺], this coordination is lost so that the heads act independently and the Vmax is equal to the ADP release rate. What makes this impossible to discern from simply examining the ATPase assays is the fact that calcium simultaneously disrupts head gating and slows ADP release. The combination of motility and ATPase measurements is necessary to demonstrate this point.

The results indicate calcium does not act as an on/off switch for motility but simultaneously reduces the ADP release rate (and thus may increase the duty cycle) and disrupts coordination between the heads of myosin VI. Whereas this will reduce the step size of myosin VI, the interesting question is what type of movement would an individual, double-headed molecule undergo on an actin filament in the presence of elevated calcium? The loss of coordination (“gating”) between the heads will undoubtedly reduce the degree of processive movement. But it is unclear whether both heads will dissociate from the actin filament, or whether the loss of coordination will cause the heads to bind and dissociate independently (and thus, the molecule will essentially walk in place, or stall, until calcium levels return to below micromolar levels). Either by dissociating or stalling the motor, processive movement would be abolished at elevated calcium concentrations. The motility of a large ensem-
ble of myosin VI HMM molecules cannot address this question. Whatever the effect of head uncoupling in vivo, it is occurring over a physiological calcium range of 1–20 μM (27).

Given that elevated calcium reduces the mantADP release rate from actomyosin VI S1 to 60% of the value without calcium (from 5.2 to 3.7 s⁻¹) and that elevated calcium reduces the motility to 60% of the value in the absence of calcium (from 131 to 80 nm s⁻¹), calcium does not affect the step size of the S1 construct. For myosin VI HMM, calcium reduces ADP release to 60% of the zero calcium value (from 5.4 to 3.6 s⁻¹) but reduces motility twice as much, to about 30% of the zero calcium value (from 307 to 103 nm s⁻¹). Filament velocity has been defined as the product of the step size and the detachment rate of the myosin head from actin (28). The detachment rate is associated with the rate-limiting step, which is ADP release for myosin VI (6). Thus, for the double-headed construct, elevated calcium would appear to reduce the step size by a factor of 2.

The simplest explanation of this (as discussed above) is that elevated calcium leads to an uncoupling of the heads, so that the heads are independent. Thus, at elevated calcium, the N-terminal lobe of CaM may detach from the calcium. Based on previous work on myosin I (29), it might be expected that the C-terminal calcium binding sites of calmodulin mediate the altered ADP release rate. However, as shown in Fig. 5, it is the N-terminal calcium binding sites of calmodulin that lead to the altered ADP release and steady state kinetics. As shown previously (12) and in the present study, calmodulin does not completely dissociate from the myosin VI heavy chain. Thus, we propose that in the presence of elevated calcium, the N-terminal lobe of CaM may detach from the IQ, whereas the C-terminal lobe remains attached. Such an interaction has been seen between one of the IQ motifs of yeast myosin V and an essential light chain (30). If calcium does cause release of part of the IQ motif from the N-terminal lobe of CaM, then this could create a flexible linker between the heads that allows the heads to act independently.

Acknowledgments—We are grateful to Dr. Graham Côté for the kind gift of PAK3. Also, we greatly appreciate the assistance of Dr. Steve Rosenfeld in providing the phosphate-binding protein, mantADP, and experimental advice. We thank J. Nolt for assistance with protein preparation.

REFERENCES
1. Wells, A. L., Lin, A. W., Chen, L.-Q., Safer, D., Cain, S. M., Hasson, T., Carragher, B. O., Milligan, R. A., and Sweeney, H. L. (1998) Nature 401, 505–508
2. Holmes, K. C., and Geeves, M. A. (1999) Annu. Rev. Biochem. 68, 687–729
3. Houssus, A., and Sweeney, H. L. (2001)Curr. Opin. Struct. Biol. 11, 182–194
4. Uyeda, T. Q., Abramson, P. D., and Spudich, J. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4459–4464
5. Purcell, T. J., Morris, C., Spudich, J. A., and Sweeney, H. L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 14159–14164
6. De La Cruz, E. M., Ostap, E. M., and Sweeney, H. L. (1999) J. Biol. Chem. 274, 32573–32581
7. Rock, R. S., Rice, S. E., Wells, A. L., Purcell, T. J., Spudich, J. A., and Sweeney, H. L. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 13655–13659
8. Nishikawa, S., Homma, K., Komori, Y., Iwaki, M., Wazawa, T., Iwane, A. H., Saito, J., Ikebe, R., Katayama, E., Yanagida, T., and Ikebe, M. (2002) Biochem. Biophys. Res. Commun. 290, 311–317
9. Veigel, C., Wang, F., Bartos, M. L., Sellers, J. R., and Mulloy, J. E. (2002) Nat. Cell Biol. 4, 59–65
10. Homma, K., Saito, J., Ikebe, R., and Ikebe, M. (2000) J. Biol. Chem. 275, 34766–34771
11. Trybus, K. M., Krementsova, E., and Freyzon, Y. (1999) J. Biol. Chem. 274, 27448–27456
12. Yoshimura, M., Homma, K., Saito, J., Inoue, A., Ikebe, R., and Ikebe, M. (2001) J. Biol. Chem. 276, 39809–39817
13. Bement, W. M., and Mooseker, M. S. (1995) Cell Motil. Cytoskeleton 31, 87–92
14. Buss, F., Kendrick-Jones, J., Lionne, C., Knight, A. E., Cote, G. P., and Luzio, P. J. (1997) J. Cell Biol. 143, 1535–1545
15. Hasson, T., and Mooseker, M. S. (1994) J. Cell Biol. 127, 425–440
16. Hopp, T. P., Prickett, K. S., Price, V., Libby, R. T., March, C. J., Cerretti, P., Urdal, D. L., and Conlon, P. J. (1988) Biotechnology 6, 1205–1210
17. Kollodziej, P. A., and Young, R. A. (1991) Methods Enzymol. 194, 508–519
18. Trybus, K. M., Freyzon, Y. Faust, L. Z., and Sweeney, H. L. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 48–52
19. Sweeney, H. L., Rosenfeld, S., Brown, F., Faust, L., Smith, J., Stein, L., and Sellers, J. (1998) J. Biol. Chem. 273, 6626–6670
20. Hiratsuka, T. (1983) Biochim. Biophys. Acta 742, 496–508
21. Pardee, J. D., and Spudich, J. A. (1982) Methods Enzymol. 85, 164–181
22. Brune, M., Hunter, J. L., Corrie, J. E., and Webb, M. R. (1994)Biochemistry 33, 8262–8271
23. Sellers, J. R., Cuda, G., Wang, F., and Homsher, E. (1993) Methods Cell Biol. 39, 25–49
24. Walker, M. I., Burgess, S. A., Sellers, J. R., Wang, F., Hammer, J. A. 3rd, Trinick, J., and Knight, P. J. (2000) Nature 405, 804–807
25. Burgess, S., Walker, M., Wang, F., Sellers, J. R., White, H. D., Knight, P. J., and Trinick, J. (2002) J. Cell Biol. 159, 983–991
26. Rice, S., Lin, A. W., Safer, D., Hart, C. L., Narber, N., Carragher, B. O., Cain, S. M., Pechatnikova, E., Wilson-Kubalek, E. M., Whittaker, M., Pate, E., Cooke, R., Taylor, E. W., Milligan, R. A., and Vale, R. D. (1999) Nature 402, 778–784
27. Mammano, F., Freidenkow, G. I., Lagostena, L., Belyantseva, I. A., Kurc, M., Dobane, V., Colavita, A., and Ruch, R. (1999) J. Neurosci. 19, 6918–6929
28. Uyeda, T. Q., Kron, S. J., and Spudich, J. A. (1990) J. Mol. Biol. 214, 699–710
29. Zhu, T., Beckingham, K., and Ikebe, M. (1998) J. Biol. Chem. 273, 20481–20486
30. Terrak, M., Wu, G., Stafford, W. F., Lu, R. C., and Dominguez, R. (2003) EMBO J. 22, 362–371