Dynamics of the Upper 50-kDa Domain of Myosin V Examined with Fluorescence Resonance Energy Transfer

Mingxuan Sun‡, Judy L. Oakes†, Shobana K. Ananthanarayanan†, Katherine H. Hawley‡, Roger Y. Tsien‡, Stephen R. Adams§, and Christopher M. Yengo不太好

From the ‡Department of Biology, University of North Carolina, Charlotte, North Carolina 28223 and the §Department of Pharmacology, University of California at San Diego, La Jolla, California 92039

The upper 50-kDa region of myosin may be critical for coupling between the nucleotide- and actin-binding regions. We introduced a tetracysteine motif in the upper 50-kDa domain (residues 292–297) of myosin V containing a single IQ domain (MV 1IQ), allowing us to label this site with the fluorescein biarsenic hairpin-binding dye (FlAsH) (MV 1IQ FlAsH). The enzymatic properties of MV 1IQ FlAsH were similar to those of unlabeled MV 1IQ except for a 3-fold reduced ADP-release rate. MV 1IQ FlAsH was also capable of moving actin filaments in the in vitro motility assay. To examine rotation of the upper 50-kDa region, we determined the difference in the degree of energy transfer from N-methylanthraniloyl (mant)-labeled nucleotides to FlAsH in both steady-state and transient kinetic experiments. The energy transfer efficiency was higher with mant-ATP (0.65 ± 0.02) compared with mant-ADP (0.55 ± 0.02) in the absence of actin. Stopped-flow measurements suggested that the energy transfer efficiency decreased with phosphate release (0.04 s⁻¹) in the absence of actin. In contrast, upon mixing MV 1IQ FlAsH in the ADP-Pi state with actin, a decrease in the energy transfer signal was observed at a rate of 13 s⁻¹, similar to the ADP release rate. Our results demonstrate there was no change in the energy transfer signal upon actin-activated phosphate release and suggest that actin binding alters the dynamics of the upper 50-kDa region, which may be critical for the ability of myosin to bind tightly to both ADP and actin.

Myosins consist of a superfamily of molecular motor proteins that use the energy from ATP hydrolysis to generate force and motion through a cyclic interaction with actin filaments (1). The structural details of how conformational changes in myosin are coupled to specific steps in the actomyosin ATPase cycle have yet to be fully elucidated. The current working model suggests that small changes in the nucleotide-binding region (switch I and switch II) are communicated to the lever arm region and amplified to generate force and motion (2). One key question that remains is how myosin changes conformation to alter its affinity for actin in a nucleotide-dependent manner. The myosin V crystal structures (3, 4) as well as previous structural and biochemical data (5–10) suggest that the large cleft that separates the actin-binding region and extends from the nucleotide-binding site may change conformation in a nucleotide-dependent manner. However, it is unclear how the coupling between the nucleotide-binding region and actin-binding cleft occurs.

The structure of myosin subfragment 1 can be described by three domains generated by trypsin cleavage: a 25-kDa N-terminal domain, a central 50-kDa domain, and a 20-kDa C-terminal domain (Fig. 1). The actin-binding domain is separated into the upper and lower 50-kDa domains by a 50-kDa cleft, also known as the actin-binding cleft. The actin-binding cleft extends from near the active site to the actin-binding region, making it a logical candidate for communication between the nucleotide- and actin-binding domains (9, 10). The atomic structures demonstrate a rotation of the upper 50-kDa region in the nucleotide-free structure of myosin V, which results in closure of the actin-binding cleft, compared with the near rigor and ATP-bound states (4). In addition, cryoelectron microscopy demonstrated a structural change in the upper 50-kDa region of smooth muscle myosin when bound to actin in the presence and absence of ADP (6). Another study demonstrated that a tryptophan residue in the upper 50-kDa region of the cleft changed conformation with kinetics identical to that of ATP-induced dissociation from actin (7). Finally, a previous study that placed pyrene probes on either side of the cleft monitored a change in pyrene excimer fluorescence upon ATP-induced dissociation from actin (8). Thus, the cleft appears to play a role in an ATP-dependent conformational change that results in a large reduction in affinity for actin. It has been proposed that the upper 50-kDa rotation occurs by distortion of a highly conserved β sheet, which may be a conformational change similar to that observed in the sequential product release of F1-ATPase (4, 11). However, little is known about how the cleft changes conformation upon binding to actin in the ADP-Pi state and if the cleft plays a role in the actin-activated P0 and ADP release. Studies in which the dynamics of the actin-binding cleft can be measured in the presence of actin during the product release steps are critical to answering these questions.

Myosin V is an actin-based molecular motor that has a long neck, containing six IQ domains, which allows it to move processively along actin (take multiple steps along actin without diffusing away) (12). Myosin V also has unique kinetic properties that make it a good candidate to examine actin-dependent conformational changes in myosin (13). First, it has a higher affinity for actin than class II muscle myosins in the weak binding prepower stroke states (14). Second, myosin V has a fast rate of actin-activated phosphate release and a slow rate of ADP release, which allows one to distinguish between conformational changes associated with the two product release steps at fairly low actin concentrations (13, 15). Finally, the predominant steady-state intermediate for myosin V is the actomyosin-ADP state (13, 16), which makes myosin V a good model to examine how myosin can bind both ADP and actin tightly.

In the current study, we introduced a tetracysteine motif in the upper 50-kDa region of myosin V, which was labeled with the fluorescein biarsenic hairpin-binding dye (FlAsH) (17, 18). We then investi-
gated the structural properties of myosin V labeled with FlAsH and measured the fluorescence resonance energy transfer (FRET) from mant-labeled nucleotides to the FlAsH site on the upper 50-kDa region of myosin V. By characterizing changes in FRET with steady-state and transient kinetic measurements, we demonstrate that the upper 50-kDa region is very sensitive to the presence of actin and that the dynamics of this region may be a critical means of communication between the nucleotide- and actin-binding regions.

**EXPERIMENTAL PROCEDURES**

**Reagents**—All reagents were the highest purity commercially available. ATP and ADP were prepared fresh from powder. N-Methylanilinyl (mant)-labeled 2'-deoxy-ATP and 2'-deoxy-ADP were prepared as described (19) or purchased from Jenna Biosciences (Jena, Germany). The mant-ATP and mant-ADP concentrations were determined from absorbance measurements at 259 nm using ε_{259} of 23,300 M^{-1}·cm^{-1}. ATP and ADP concentrations were determined by absorbance at 259 nm using ε_{259} of 15,400 M^{-1}·cm^{-1}. Nucleotides were prepared prior to use in the presence of equimolar MgCl₂.

**Myosin cDNA Construction and Protein Expression and Purification**—Site-directed mutagenesis was performed on a construct of chicken myosin V containing a single IQ motif (WT MV 1IQ) (residues 1–792). We substituted residues (positions 292–297) with a tetracysteine motif (Cys-Cys-Pro-Gly-Cys-Cys) to generate the MV 1IQ construct for fluorescent labeling. The baculovirus system was used to express MV 1IQ, which contained a C-terminal Myc tag (EQKLISEEDL) followed by a FLAG tag (DYKDDDK) for purification purposes (13–16). All myosin V constructs were coexpressed with chicken calmodulin. The purity was greater than 95% based on Coomassie-stained SDS gels. Myosin concentrations were determined using the Bio-Rad microplate assay using bovine serum albumin as a standard (14, 15). Actin was purified from rabbit skeletal muscle using an anion-exchange chromatography method (21). All experiments were performed in KMg50 TCEP buffer (50 mM KCl, 1 mM EGTA, 1 mM MgCl₂, 1 mM TCEP, and 10 mM imidazole-HCl, pH 7.0, 25 °C).

**Labeling Myosin V 1IQ with FlAsH**—A 2.5 μM stock of MV 1IQ, containing the tetracysteine motif and referred to as MV 1IQ hereafter, in KMg50 TCEP was incubated with 10 μM FlAsH for 20 min at room temperature and then left on ice for 12–14 h. The FlAsH-labeled MV 1IQ was then dialyzed for 24 h in KMg50 TCEP to remove the excess dye. The MV 1IQ labeled with FlAsH, referred to as MV 1IQ FlAsH, was examined by SDS-PAGE, using a modified Laemmli buffer (18). The extent of labeling of MV 1IQ (unlabeled) was examined by measuring FlAsH by absorbance (ε_{600} = 63,500 M^{-1}·cm^{-1}) and MV 1IQ by the method of Bradford as described above and single turnover measurements.

**Steady-state ATPase Activity of MV 1IQ**—Steady-state ATP hydrolysis by MV 1IQ constructs (50–100 nM) in the absence and presence of actin (0–40 μM) was examined using the NADH-linked assay (13–16) with a final MgATP concentration of 1 mM.

**In Vitro Motility Assay**—Actin filament motility was measured using an in vitro motility assay method described previously (22). Briefly, a 10-μl flow cell was constructed using a nitrocellulose-coated coverslip supported by two strips of double-sided Scotch tape. The myosin was specifically attached to the nitrocellulose-coated surface with an anti-Myc antibody and blocked with bovine serum albumin at a concentration of 1 mg/ml. The motility of F-actin labeled with rhodamine-phaloidin was observed using an activation buffer consisting of KMg50, 3.4 μM calmodulin, and 1 mM ATP. Phosphoenol pyruvate (2.5 mM) and pyruvate kinase (20 units/ml) were added as an ATP regeneration system, and glucose oxidase (0.1 mg/ml), glucose (5 mg/ml), and catalase (0.018 mg/ml) were added to reduce photobleaching. After the addition of the activation buffer, the slide was promptly viewed using an inverted epifluorescent microscope (Olympus IX70) under ×100 magnification. Real-time video images were obtained using a Hamamatsu C8484-05G camera (Hamamatsu Photonics, Hamamatsu, Japan). To measure velocity, the video records were transferred to the Metamorph Imaging System (Universal Imaging Corp., Downingtown, PA).

**Fluorescence Spectroscopy**—A Quantamaster fluorometer (Photon Technology International, Lawrenceville, NJ), equipped with a 75-watt xenon arc lamp as an excitation source and excitation/emission monochrometers, was used to measure steady-state fluorescence of mant-labeled nucleotides and MV 1IQ FlAsH. Mant-labeled nucleotides were excited at 365 nm, and the emission spectra were measured from 400 to 550 nm. FlAsH was excited directly at 495 nm, and the emission was measured from 500 to 600 nm. Steady-state energy transfer measurements were performed by exciting mant-labeled nucleotides at 365 nm, and the emission was measured from 400 to 600 nm. Slit widths were set at a resolution of 2 nm. All fluorescence spectra were corrected for variations in the wavelength sensitivity of the detector system and the presence of Raman scatter and background fluorescence in the appropriate buffer solution.

Steady-state anisotropy measurements were taken using excitation and emission Glann Thompson polarizers. Anisotropy was calculated following correction for the G-factor at the appropriate wavelength (24). Anisotropy measurements were performed at 25 °C in KMg50 TCEP buffer. In addition, steady-state anisotropy measurements were performed in KMg50 TCEP buffer containing 50% glycerol at −13 °C to estimate the fundamental anisotropy (r₀).  

**FRET Measurements**—FRET was used to measure the distance between donor fluorophore, mant-labeled nucleotides, and the acceptor fluorophore, FlAsH-labeled MV 1IQ, using the Förster energy transfer theory (23, 24). The efficiency (E) of energy transfer was measured by examining the increase in the acceptor emission. The efficiency of transfer is given by the following:

\[
E = \frac{\varepsilon_A(\lambda^{em}_A)}{\varepsilon_D(\lambda^{em}_D)} \left[ \frac{F_{AD}(\lambda^{em}_A)}{F_A(\lambda^{em}_A)} - 1 \right] \left( \frac{1}{F_D} \right) 
\]

where \( F_A(\lambda^{em}_A) \) represents the fluorescence intensity at the emission peak of the acceptor in the absence of the donor, \( F_{AD}(\lambda^{em}_A) \) is the fluorescence intensity from the acceptor in the presence of the donor, corrected by subtracting the fluorescence from unbound mant nucleotides,
and \(\varepsilon_{365}\) and \(\varepsilon_{366}\) are the extinction coefficients of the acceptor (1259 M\(^{-1}\)cm\(^{-1}\)) and donor (5304 M\(^{-1}\)cm\(^{-1}\)) at 365 nm. The fraction of mant nucleotide bound to myosin \(f_d\) was determined from the dissociation constant, which was measured by fluorescence titration (data not shown).

The Förster distance at which energy transfer is equal to 50% \(R_0\) was calculated from Equation 2,

\[
R_0 = \left(\frac{8.79 \times 10^{23}}{4} \eta^{-2} Q_d J(\lambda) \right)^{1/6}
\]

(Eq. 2)

where \(\eta\) represents the refractive index of the buffer (\(\eta = 1.4\)), \(Q_d\) is the quantum yield of mant-labeled nucleotides (0.51, 0.46, and 0.46 for mant-ATP bound to MV 1IQ and mant-ADP bound to MV 1IQ and acto-MV, respectively), \(\kappa^2\) is the orientation factor, which ranges from 0 to 4 and is 0.67 when there is free rotation of the donor and acceptor fluorophores, and \(J\) is the spectral overlap integral \(\left(f = 6.13 \times 10^{-15}\right)\), determined from Equation 3. The quantum yields were determined with a comparative method using mant-ATP alone as a standard \((Q_d = 0.22)\) (25).

\[
J(\lambda) = \int \varepsilon_A(\lambda) \cdot F_D(\lambda) \cdot \lambda^4 d\lambda
\]

(Eq. 3)

In Equation 3, \(F_D(\lambda)\) represents the normalized total integrated intensity of the unquenched donor group, and \(\varepsilon_A(\lambda)\) is the extinction coefficient of the acceptor. \(J\) was numerically integrated at 1-nm intervals, and \(\lambda\) is the wavelength in centimeters. The distance \(r\) between the donor and acceptor fluorophores was calculated with Equation 4.

\[
r = R_0 \left(1 - E/E\right)^{1/6}
\]

(Eq. 4)

Stopped-flow Measurements and Kinetic Modeling—Transient kinetic experiments were performed in an Applied Photophysics (Surrey, UK) stopped-flow apparatus with a dead time of 1.2 ms. A monochromator with a 2-nm band pass was used for fluorescence excitation, and cut-off filters were used to measure the emission. All optical filters were provided with the stopped-flow instrument unless otherwise indicated. Pyrene actin and mant-labeled nucleotides were excited at 365 nm, and the emission was measured using a 400-nm long pass filter. FlAsH fluorescence was directly excited at 495 nm or measured by energy transfer from mant-labeled nucleotides by exciting at 365 nm, and the emission was measured with a 515-nm long pass filter. We isolated the mant fluorescence signal in the FRET experiments by using both a 400-nm long pass filter and a 500-nm short pass filter (03SWP406; Melles Griot, Carlsbad, CA). Nonlinear least-squares fitting of the data was done with software provided with the instrument or Kaleidagraph (Synergy Software, Reading, PA). Uncertainties reported are S.E. of the fits unless stated otherwise.

Kinetic modeling and simulations were performed with Pro-K software (Applied Photophysics) using the reaction scheme that has been used in recent kinetics studies of myosin V (13–16). All concentrations mentioned in the stopped-flow experiments are final concentrations unless stated otherwise.

RESULTS

Labeling with FlAsH—Our results demonstrate 1:1 stoichiometric and specific labeling of MV 1IQ, containing the tetracysteine motif, with FlAsH. Fig. 2 demonstrates the labeling of MV 1IQ following SDS-PAGE. The fluorescent myosin heavy chain band from the MV 1IQ construct was compared with WT MV 1IQ incubated for 14 h with FlAsH. The results clearly demonstrate that FlAsH only labeled the MV 1IQ, which contains the tetracysteine motif, and did not bind WT MV 1IQ. The degree of labeling of MV 1IQ FlAsH was measured by determining the FlAsH concentration by absorbance. Bradford assays were used to determine the MV 1IQ concentration, which was verified by single turnover experiments (see below). These results are consistent with the results of Adams et al. (18) that demonstrated an affinity of FlAsH for tetracysteine peptides in the picomolar range.

Functional Assays—We first measured the ATPase activity of MV 1IQ to determine if introduction of the tetracysteine motif altered the activity of myosin V. The ATPase activity of MV 1IQ (unlabeled) was similar to that of MV 1IQ (WT) (Table 1) (13) but with a slightly higher \(V_{\text{max}}\). The steady-state ATPase measurements demonstrate that the MV 1IQ FlAsH has a 2–3-fold reduced maximal rate of ATPase \((V_{\text{max}})\) but a similar actin concentration at which the ATPase activity is half-maximal \(K_{\text{ATPase}}\) (Fig. 3A and Table 1). There was no difference between the basal ATPase rate of MV 1IQ (unlabeled), MV 1IQ FlAsH, and MV 1IQ WT. In addition, single turnover measurements (data not shown) demonstrated that the ATPase rates in the absence of actin were similar to the basal rates measured in the NADH-coupled assay. Thus, nearly 100% of the purified myosin was active, and the concentration determined by the method of Bradford was validated.

To determine if the reduction in the \(V_{\text{max}}\) of MV 1IQ FlAsH was a result of a reduced ADP release rate, we directly examined the rate of ADP release from MV FlAsH in the presence of actin (Fig. 3B). The rate of ADP release, measured by mixing MV 1IQ (0.25 \(\mu\)M) bound to pyrene actin (0.25 \(\mu\)M) and ADP (20 \(\mu\)M) with excess ATP (5 mM), was similar to the \(V_{\text{max}}\) for both MV 1IQ (unlabeled) (22.2 \(\pm\) 0.2 s\(^{-1}\)) and MV 1IQ FlAsH (8.6 \(\pm\) 0.2 s\(^{-1}\)). Thus, ADP release is probably the rate-limiting step in the ATPase cycle of MV 1IQ (unlabeled) and MV 1IQ FlAsH.

The results of the \textit{in vitro} motility assay demonstrated a 20% reduction in the actin filament velocity generated by MV 1IQ FlAsH compared with MV 1IQ (unlabeled), and MV 1IQ unlabeled was similar to WT MV 1IQ (Table 1).

Steady-state Fluorescence—We examined the degree of FRET from mant-ATP or mant-ADP to the FlAsH site upon binding of these fluorescent nucleotides to MV 1IQ FlAsH (Fig. 4, A and B). The enhancement of the FlAsH fluorescence was used to monitor the energy transfer...
presence of mant-ATP, mant-ADP, and acto-MV 1IQ FlAsH was state anisotropy measurements (excitation 365 nm; emission 445 nm) of equation, plotted as a function of mant-ATP or mant-ADP and fit to the binding equation, and the results are shown in Table 1.

The resulting distance (r) associated with the MV 1IQ FlAsH in the presence of mant-ATP, mant-ADP, and acto-MV 1IQ FlAsH was 25.2 ± 0.4, 27.0 ± 0.2, and 27.5 ± 1.2 Å, respectively (Table 2).

**Steady-state Anisotropy Measurements**—We found that the steady-state anisotropy measurements (excitation 365 nm; emission 445 nm) of MV 1IQ/mant-ATP, MV 1IQ/mant-ADP, and acto-MV 1IQ/mant-ADP were quite similar (r = 0.19 ± 0.01, 0.17 ± 0.01, and 0.17 ± 0.01, respectively). The ratio of myosin V to mant-ATP or mant-ADP was adjusted to ensure that the nucleotides were >95% bound (2 μM MV 1IQ (unlabeled)/1 μM mant-ATP, 14 μM MV 1IQ (unlabeled)/1 μM mant-ADP, and 14 μM acto-MV 1IQ (unlabeled)/1 μM mant-ADP). The steady-state anisotropy (excitation 500 nm; emission 528 nm) of 0.5 μM MV 1IQ FlAsH was also measured (r = 0.28 ± 0.01). The fundamental anisotropy (r_0) determined at -13 °C in KMG50 buffer containing 50% glycerol was found to be similar for mant-ATP (r_0 = 0.33 ± 0.01) and mant-ATP (r_0 = 0.32 ± 0.01) bound to MV 1IQ (unlabeled). The fundamental anisotropy of MV 1IQ FlAsH was determined to be 0.35 ± 0.01.

**Transient Kinetic Measurements**—The rates of mant-ATP and mant-ADP binding to MV FlAsH were monitored by FRET by examining the fluorescence increase in the acceptor (FlAsH) (excitation 365 nm; emission 515 nm; LP filter) upon mixing with mant-ATP or mant-ADP in the stopped flow (Fig. 6). The second-order rate constant for mant-ATP binding to MV 1IQ FlAsH was 0.93 ± 0.04 μM^{-1}s^{-1}, whereas the second-order rate constant for mant-ATP binding to acto-MV 1IQ FlAsH or MV 1IQ FlAsH was 10.1 ± 0.3 and 10.1 ± 0.6 μM^{-1}s^{-1}, respectively. The rate of ADP release was monitored by mixing MV 1IQ FlAsH in the presence of actin (Fig. 7). We performed sequential mix experiments to measure the FRET signal (excitation 365 nm; emission 515 nm; LP filter) during phosphate and ADP release in the absence of actin (Fig. 7). We mixed MV 1IQ FlAsH with limiting mant-ATP, allowed the reaction to age for 5 or
Summary of energy transfer results

| Nucleotide state                  | FRET efficiency * | R₀[Å] | r(2/3) |
|----------------------------------|------------------|-------|--------|
| MV 1IQ FlAsH/mant-ATP            | 0.65 ± 0.02      | 28.0  | 25.2 ± 0.4 |
| MV 1IQ FlAsH/mant-ADP            | 0.55 ± 0.02      | 27.5  | 27.0 ± 0.2 |
| Acto-MV 1IQ FlAsH/mant-ADP       | 0.61 ± 0.06      | 27.5  | 25.5 ± 1.0 |

* FRET efficiency with 100% bound mant-ATP or mant-ADP calculated from the titration curve shown in Fig. 5.

** Förster distance at which the efficiency of energy transfer is 50%.

*** Distance (r) between the donor and acceptor probes calculated using a r² value of 2/3.

120 s, and then mixed with saturating ADP (final concentrations: 0.20 μM MV 1IQ FlAsH, 0.15 μM mant-ATP, and 1 mM ADP). The fluorescence transient after a 5-s age time followed a single exponential (0.040 ± 0.001 s⁻¹). The fluorescence transient after a 120-s age time followed a biexponential decay (9.9 ± 2 and 0.035 ± 0.001 s⁻¹; fractional amplitudes of 0.75 and 0.25, respectively). The relative concentrations of M·ADP-P, and M·ADP (0.19 and 0.81, respectively) following the 120-s lag were determined by simulating the data to a kinetic model. Comparing the relative amplitudes of the 120-s lag trace and the simulated data allows us to estimate that the FRET signal in the M·ADP-P state is 40% enhanced compared with the M·ADP state.

We also performed sequential mix experiments to monitor the FRET signal (excitation 365 nm; emission 515 nm; LP filter) upon actin-activated phosphate-release and ADP-release (Fig. 8). MV 1IQ FlAsH was mixed with mant-ATP, allowed to age for 2 s, and then mixed with actin (final concentrations, 20 μM actin, 0.25 μM FlAsH, and 0.2 μM mant-ATP). The fluorescence of FlAsH decreased and was fit to a single exponential rate constant of 13.7 ± 0.3 s⁻¹. The same experiment was performed examining the mant fluorescence signal (excitation 365 nm; emission 400 nm; LP filter), and the resulting fluorescence decrease was biphasic and fit to two exponentials, 195 ± 14 and 13.7 ± 0.5 s⁻¹. Similar results were obtained by monitoring the mant fluorescence signal (excitation 365 nm; emission 400 nm; LP filter) (210 ± 7.0 and 20 ± 1.0 s⁻¹) with myosin V 1IQ. We also performed the experiment with MV 1IQ FlAsH and monitored the mant fluorescence signal by using a second filter to block the FlAsH signal (excitation 365 nm; emission 400 nm; 400LP/500SP filter) and obtained a rate of 220 ± 13 s⁻¹ for the fast phase (data not shown).

**DISCUSSION**

The results of the functional assays demonstrate that labeling myosin V with FlAsH has a minimal affect on the functional properties of myo-
Dynamics of the Upper 50-kDa Domain of Myosin

![Graph showing measurement of FRET during the actin-activated product release steps.](Image)

The results are from the following sequential mix experiments. MV 1IQ (unlabeled) (A) or MV 1IQ FlAsH (1.0 μM) (B) was mixed with limiting mant-ATP (0.8 μM), allowed to age for 2 s, and then mixed with actin (40 μM) (concentrations in the final mix: 0.25 μM MV FlAsH, 0.2 μM mant-ATP, and 20 μM actin). The FRET signal was isolated by using a 515 nm long pass emission filter (trace 2, bottom), and the mant signal was monitored using a 400 nm long pass filter (trace 1, top). Each fluorescence (arbitrary units (AU)) trace includes the raw data and the fit of the data, and the increased noise observed in the first 0.1 s is a result of more data points being collected during this time period.

The energy transfer results suggest that there is a difference in the position of the upper 50-kDa domain in the ATPase cycle of myosin V. Indeed, MV 1IQ FlAsH retained its ability to translocate actin filaments in the in vitro motility assay. Thus, the FlAsH-labeled myosin V is a functional molecular motor and an excellent model to measure conformational changes in myosin. Our FlAsH labeling results suggest that a hairpin structure is not critical for tight binding of the bisarennesal probe, since the tetracysteine motif was placed in a surface loop of myosin V (3, 4). Our study adds to other recent studies that used the tetracysteine/FlAsH-based system for fluorescent labeling of proteins (26, 27) and confirms that this is a powerful method of performing structure/function studies.

Our energy transfer results suggest that there is a difference in the position of the upper 50-kDa domain in the ATPase cycle of myosin V except for a reduction in ADP release rate, the rate-limiting step in the ATPase cycle of myosin V. Indeed, MV 1IQ FlAsH retained its ability to translocate actin filaments in the in vitro motility assay. Thus, the FlAsH-labeled myosin V is a functional molecular motor and an excellent model to measure conformational changes in myosin. Our FlAsH labeling results suggest that a hairpin structure is not critical for tight binding of the bisarennesal probe, since the tetracysteine motif was placed in a surface loop of myosin V (3, 4). Our study adds to other recent studies that used the tetracysteine/FlAsH-based system for fluorescent labeling of proteins (26, 27) and confirms that this is a powerful method of performing structure/function studies.

Our transient kinetic experiments monitoring the FRET signal also demonstrate that the conformation of the upper 50-kDa region is sensitive to the presence of the γ-phosphate of ATP. The sequential mix experiments in the absence of actin clearly demonstrate a decrease in the FRET signal at a rate of 0.04 s⁻¹, similar to the phosphate release rate, whereas in the presence of actin, myosin mainly populates the closed nucleotide-binding cleft state (high energy transfer) (2). A similar rate (0.05 s⁻¹) was determined from the steady state ATPase measurements (Table 1). In addition, the results of the relative amplitudes of the fast (ADP release) and slow (Pi release) phases following the 120-s age time further suggest that the FRET signal decreases during phosphate release.

In contrast to the absence of actin, our results suggest that the upper 50-kDa domain remains in a similar conformation upon actin-activated phosphate release. Our results from mixing MV 1IQ FlAsH in the ADP-P state with actin demonstrate that the fluorescence transient of the FRET signal decreases at a rate of 13 s⁻¹, similar to the rate of mant-ADP release. We also examined the mant fluorescence transient with the same experimental set up and observed a biexponential decrease in mant fluorescent (190 and 13 s⁻¹). These results are similar to a study by Sweeney and Rosenfeld (30), which demonstrated that the fast phase of the mant fluorescence signal was equivalent to the phosphate release rate, whereas the slow rate was similar to the ADP release rate. Thus, the FRET signal appears to monitor the actin-activated ADP release but is insensitive to phosphate release. Interestingly, since the quantum yield of the donor decreases with phosphate release but the FRET efficiency...
does not change, there may be a small decrease in the distance between the probes upon phosphate release.

The data from this study indicate that the dynamics of the upper 50-kDa domain are altered by the presence of actin, and actin-activated phosphate release may occur through a mechanism other than rotation of the upper 50-kDa domain. Two models have been proposed to explain how product release occurs in myosin. The first model suggests that the movements in switch I are responsible for the release of phosphate (31, 32). Since the crystal structures of myosin V demonstrate that movement of switch I is associated with the rotation of the upper 50-kDa region, our results may not support this model. However, it is possible that there is a unique rotation of the upper 50-kDa domain upon actin-activated phosphate release that our probes are not sensitive to. Interestingly, our results may suggest that the nucleotide-binding pocket remains closed following actin-activated phosphate release, whereas a unique twist of the so-called transducer region (seven-stranded β sheet) (3) allows closure of the actin-binding cleft and strong binding to actin. This would allow myosin to bind tightly to ADP and actin following phosphate release. The second model suggests that phosphate release occurs through the “back door” mechanism, in which movements of the upper 50-kDa region may not be necessary for phosphate release. Thus, our results support a model of actin-activated phosphate release occurring by a back door mechanism. Other support for the back door mechanism has been provided by attempts to sterically block the back door (33) as well as by molecular dynamics simulations (34). The structural change we have measured in the upper 50-kDa domain may indicate that rotation of this domain occurs to allow phosphate release in the absence of actin, but in the presence of actin interactions of this domain with actin prevent its rotation, and thus phosphate release may occur by a unique conformational change in this region or an alternative mechanism.

Force generation is thought to occur during the actin-activated phosphate release step in myosin (35). Thus, it is critical to understand how phosphate release triggers the structural change in the lever arm, while myosin is strongly bound to actin. Our results suggest the rotation of the upper 50-kDa domain may not play a role in this force-generating structural mechanism. However, movements of the lower 50-kDa domain may be responsible for the structural communication from the active site to the lever. Indeed, it has been demonstrated that in the absence of actin, the formation of the pre-power stroke state is associated with movements in the switch II region and rotation of the lower 50-kDa domain (2). It is still unclear whether the reversal of this conformational change is responsible for the force-producing lever arm swing or if other structural changes not yet revealed may be mediating this important structural change.

In summary, we have demonstrated that the biarsenical/tetracysteine fluorescence labeling system is an extremely powerful method for measuring protein domain motions via energy transfer. Our FRET results demonstrate that the upper 50-kDa region of myosin is sensitive to the nucleotide state and the presence of actin. In addition, our results suggest that the upper 50-kDa domain may undergo a unique conformational change or remain in a specific conformation during actin-activated phosphate release. The unique conformation of the upper 50-kDa region in the presence of actin and nucleotide may be critical for allowing myosin to bind both ADP and actin tightly.

Acknowledgments—We thank Dr. Lee Sweeney for providing the initial mant-ADP sample. We also thank Dr. Christopher Berger and Dr. Carl Morris for helpful discussions.

REFERENCES

1. Sellers, J. R. (1999) Myosins, 2nd Ed., Oxford University Press, Oxford, UK
2. Geeves, M. A., and Holmes, K. C. (1999) Annu. Rev. Biochem. 68, 687–728
3. Coureux, P., Wells, A. L., Menetrey, J., Yengo, C. M., Morris, C. A., Sweeney, H. L., and Houdusse, A. (2003) Nature 425, 419–423
4. Coureux, P., Sweeney, H. L., and Houdusse, A. (2004) EMBO J. 23, 4527–4537
5. Holmes, K. C., and Schroeder, R. R., Sweeney, H. L., and Houdusse, A. (2004) Philos. Trans. R. Soc. Lond. B Biol. Sci. 359, 1819–1828
6. Volkman, N., Hanein, D., Ouyang, G., Trybus, K. M., DeRosier, D. J., and Lowey, S. (2001) Nat. Struct. Biol. 12, 1147–1155
7. Yengo, C. M., De La Cruz, E. M., Chrin, L. R., Gaffney, D. P., II, and Berger, C. L. (2002) J. Biol. Chem. 277, 24114–24119
8. Conibear, P. B., Bagshaw, C. R., Fajer, P. G., Kovacs, M., and Malnasi-Czismadia, A. (2003) Nat. Struct. Biol. 10, 831–835
9. Rayment, I., Rypniewski, W. R., Schmidt-Base, K., Smith, R., Tomchick, D. R., Benning, M. M., Winkelman, D. A., Wesenberg, G., and Holdem, H. M. (1993) Science 261, 50–58
10. Rayment, I., Holden, H. M., Whitaker, M., Yohn, C. B., Lorenz, M., Holmes, K. C., and Milligan, R. A. (1993) Science 261, 58–65
11. Menz, R. L., Walker, J. E., and Leslie, A. G. (2001) Cell 103, 331–341
12. Mehta, A. D., Rock, R. S., Rief, M., Spudich, J. A., Mooseker, M. S., and Cheney, R. E. (1999) Nature 400, 590–593
13. De La Cruz, E. M., Wells, A. L., Rosenfeld, S. S., Ostap, E. M., and Sweeney, H. L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13726–13731
14. Yengo, C. M., De La Cruz, E. M., Safer, D., Ostap, E. M., and Sweeney, H. L. (2002) Biochemistry 41, 8508–8517
15. Yengo, C. M., and Sweeney, H. L. (2004) Biochemistry 43, 2605–2612
16. De La Cruz, E. M., Sweeney, H. L., and Ostap, E. M. (2000) Biophys. J. 79, 1524–1529
17. Griffin, B. A., Adams, S. R., Jones, J., and Tsien, R. Y. (2000) Methods Enzymol. 327, 585–578
18. Adams, S. R., Campbell, R. E., Gross, L. A., Martin, B. R., Walkup, G. K., Yao, Y., Llopis, J., and Tsien, R. Y. (2002) J. Am. Chem. Soc. 124, 6063–6076
19. Hiratsuka, T. (1983) Biochim. Biophys. Acta 742, 496–508
20. Pardee, J., and Spudich, J. A. (1982) Methods Enzymol. 85, 164–181
21. Pollard, T. D. (1984) J. Cell Biol. 99, 769–777
22. Kron, S. J., Toyoshima, Y. Y., Uyeda, T. Q., and Spudich J. A. (1991) Methods Enzymol. 196, 399–416
23. Clegg, R. M. (1992) Methods Enzymol. 211, 353–388
24. Lakowicz, J. R. (1999) Principles of Fluorescence Spectroscopy, 2nd Ed., Kluwer Academic/Plenum Press, New York
25. Yengo, C. M., Chrin, L. R., Rovner, A. S., and Berger, C. L. (2000) J. Biol. Chem. 275, 25481–25487
26. Robia, S. L., Flohr, N. C., and Thomas, D. D. (2005) Biochemistry 44, 4302–4311
27. Ignatova, Z., and Gierasch, L. M. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 523–528
28. Bauer, C. R., Kuhlman, P. A., Bagshaw, C. R., Rayment, I. (1997) J. Mol. Biol. 274, 394–407
29. Rosenfeld, S. S., Xing, J., Rener, B., Lebowitz, J., Kar, S., and Cheung, H. C. (1998) J. Biol. Chem. 269, 30187–30194
30. Yengo, C. M., Sweeney, H. L. (2005) J. Biol. Chem. 280, 6072–6079
31. Holmes, K. C., Angert, I., Kull, F. J., Jahn, W., and Schroeder, R. R. (2003) Nature 425, 423–427
32. Conibear, P. B., Malnasi-Czismadia, A., and Bagshaw, C. R. (2004) Biochemistry 43, 15404–15417
33. Tiago, T., Aureliano, M., and Gutierrez-Merino, C. (2004) Biochemistry 43, 5551–5561
34. Lawson, J. D., Pate, E., Rayment, I., and Yount, R. G. (2004) Biophys. J. 86, 3794–3803
35. Takagi, Y., Shuman, H., and Goldman, Y. E. (2004) Philos. Trans. R. Soc. Lond. B Biol. Sci. 359, 1913–1920