Published in final edited form as:

Nature. 2005 June 9; 435(7043): 779–785. doi:10.1038/nature03592.

The structure of the myosin VI motor reveals the mechanism of directionality reversal

Julie Ménétrey^{*,#}, Amel Bahloul^{*,#}, Amber Wells[†], Christopher Yengo[†], Carl Morris[†], H. Lee Sweeney[†], and Anne Houdusse^{*}

^{*} Structural Motility, Institut Curie CNRS, UMR144, 26 rue d'Ulm, 75248 Paris cedex 05, France

[†] Department of Physiology, University of Pennsylvania School of Medicine, 3700 Hamilton Walk, Philadelphia, PA 19104-6085 USA

Abstract

We have solved a 2.4Å structure of a truncated version of the reverse direction myosin motor, myosin VI, that contains the motor domain and binding sites for two calmodulins. The structure reveals only minor differences in the motor domain as compared to plus-end directed myosins, with the exception of two unique inserts. The first insert is near the nucleotide-binding pocket, and alters the rates of nucleotide association and dissociation. The second unique insert forms an integral part of the myosin VI converter domain along with a calmodulin bound to a previously unseen binding motif within the insert. This serves to redirect the effective “lever arm” of myosin VI, which includes a second calmodulin bound to an “IQ motif,” towards the pointed (–) end of the actin filament. This repositioning largely accounts for the reverse directionality of this class of myosin motors. We propose a model incorporating a kinesin-like uncoupling/docking mechanism to fully explain the movements of myosin VI.

The myosin superfamily is composed of eighteen classes of molecular motor proteins, the vast majority of which traffic toward the barbed (+) end of actin filaments¹. Class VI myosins were the first of the superfamily identified to traffic toward the pointed (–) end of the actin filament². They function in a number of critical intracellular processes such as vesicular membrane traffic, cell migration, maintenance of stereocilia and mitosis^{3–6}.

The current view of how myosin motors couple ATP hydrolysis and actin binding to movement is known as the lever arm hypothesis⁷. In essence the proposed mechanism is that nucleotide binding, hydrolysis and product release are all coupled to small movements within the myosin motor core. These movements are amplified and transmitted *via* a region that has been termed the “converter” domain to a lever arm consisting of a target helix and associated light chains/calmodulins. The lever arm further amplifies the motions of the converter domain into large directed movements. Consistent with the lever arm hypothesis, the stroke size has been shown to be proportional to the lever arm length^{8,9,10}. In the absence of actin, ATP hydrolysis occurs, but product release is slow, thus trapping the lever arm in a primed or pre-powerstroke position.

Correspondence should be addressed to either: Anne Houdusse, Structural Motility, UMR 144 Institut Curie-CNRS, 26 rue d'ULM, 75248 Paris cedex 05, France, Tel. 33-1-42-34-63-95, Fax. 33-1-42-34-63-82, Anne.Houdusse@curie.fr or H. Lee Sweeney, Dept. of Physiology, University of Penn., A700 Richards Bldg., 3700 Hamilton Walk, Philadelphia, PA 19104-6085, Tel. 215-898-8727, Fax. 215-573-2273, Lsweeney@mail.med.upenn.edu.

[#]Note that the first two authors contributed equally to this work.

Correspondence and requests for materials should be addressed to A.H. (anne.houdusse@curie.fr). Atomic coordinates and structure factors have been deposited in the Protein Data Bank under the accession number (2BKH and r2bkhsf) for MD^{ins2} and (2BKI and r2bkisf) for the long MD^{ins2}IQ.

Binding to actin causes release of products, plus-end-directed movement of the lever arm, and force generation concomitant with formation of strong binding between myosin and actin.

Perhaps because of their reverse directionality, myosin VI motors have a number of additional unusual features. First, the motor domain itself contains two inserts that are unique within the myosin superfamily. The first is near the nucleotide-binding pocket and the second is between the converter and IQ motif. (These correspond to residues C278-A303 and P774-Y812, respectively and are called insert-1 and insert-2 hereafter.) Single molecules of dimeric myosin VI, like myosin V, are capable of taking multiple steps (processive movement) of 30–36nm *via* a hand-over-hand mechanism along actin filaments^{11,12}. This large step size is surprising within the context of the lever arm hypothesis, in that myosin VI binds only two calmodulins per head¹³, while myosin V binds six. Furthermore, a construct truncated after the binding site for the second CaM (“IQ motif”) has a stroke (non-processive displacement upon an actin encounter) of 12nm, less than half of the step size of the two-headed molecule¹⁴. Thus the true composition of the myosin VI “lever arm” is unclear.

The fact that myosin VI binds two calmodulins per head was surprising since there is only one conventional calmodulin-binding site (IQ motif) per head¹³. It initially was postulated that the unique insert-2 forms part of the “converter” of myosin VI and redirects the lever arm (i.e. IQ-bound CaM) toward the minus end of an actin filament². That hypothesis was brought into question by studies on chimeric molecules of myosin V and VI, in which removal or addition of insert-2 did not alter directionality¹⁵. The finding that insert-2 provides a second calmodulin binding site¹³ raises the possibility that its purpose could be to simply lengthen the lever arm. In order to gain insight into the function of these two unique inserts within the myosin VI motor, we determined the crystal structure of two fragments of myosin VI.

RESULTS

Nucleotide-free myosin VI displays partial closure of the actin-binding cleft

We co-expressed calmodulin with porcine myosin VI constructs coding for either the motor domain (containing inserts 1 and 2) alone (aa. 1-816; called hereafter MD^{ins2}) or the motor domain and the helical calmodulin-binding domain (“IQ” motif) (aa. 1-859; called hereafter MD^{ins2}IQ) in insect SF9 cells. Crystals were obtained in the absence of nucleotide and diffracted to 2.4Å and 2.9Å for the MD^{ins2} and MD^{ins2}IQ constructs, respectively. The two refined structures obtained from different crystal forms superimpose very well with an r.m.s. deviation of 0.694Å for 787 Cα atoms of the motor domain (G4-Y812). These structures thus define the nucleotide-free state of myosin VI, which greatly differs from that found for plus-end myosin motors by the reversed-orientation of the lever arm (Fig. 1). The overall conformation of the myosin VI motor domain is however similar to that recently presented for myosin V¹⁶ and for the *Dictyostelium discoideum* myosin II motor in the absence of nucleotide¹⁷: the twist of the central β-sheet in all these structures greatly differs from that found for the post-rigor (ATP-) and pre-powerstroke (ADP.P_i-) states of the motor^{16,17,18}. The myosin V structure has been suggested to be in a “rigor-like” state, meaning that it is the state that myosin populates when bound to actin in the absence of nucleotide^{16,19}. Several differences – which are minor in amplitude but not in significance – distinguishes the myosin VI and II¹⁷ nucleotide-free structures from the myosin V rigor-like state. The major cleft in the molecule that closes upon binding to actin is not completely closed in myosin VI, as it is for myosin V (Supplementary movie S1). Moreover, the set of interactions found between the nucleotide-binding elements in the rigor-like state¹⁸ are also not found in myosin VI, in relation with differences in the distortion of the central β-sheet (Supplementary movie S2). The kinetics of its interaction with actin²⁰ also demonstrates that in the absence of nucleotide and actin, myosin VI does not populate the rigor-like state. Docking of the nucleotide-free myosin VI structure into reconstructions from electron microscopy images of the acto-myosin VI rigor

complex² confirms however that the state we crystallized is close to the conformation of myosin VI at the end of its powerstroke (Fig. 1a).

The role of insert-1 in modulating nucleotide association and dissociation

Myosin VI not only moves in the reverse direction, but has unusual kinetic properties. Most notable is the slow rate (40-fold slower than myosin V) of ATP-induced release of myosin VI from the actomyosin complex^{20,21}. This is primarily due to a weak affinity for ATP, which is weaker than the affinity of either myosin VI or actomyosin VI for ADP (see Supplementary Table S1). The first of the two unique inserts in myosin VI (C278-A303) belongs to the U50kDa subdomain and is located close to the nucleotide binding site, near Switch I (Fig. 1· 2a). Switch I plays a critical role in binding both the Mg²⁺ ion and the γ -phosphate of ATP in the active site. It is now believed that sequential conformational rearrangements of switch I are essential to control nucleotide release and binding from the motor^{22,23}. The location of insert-1 leads to the prediction that it may have evolved to provide unique kinetic characteristics that are potentially important for a reverse-directed motor.

To test the influence of insert-1 on the kinetics of this reverse-direction motor, we have engineered a myosin VI lacking insert-1 (Δ C278-A303) and measured the impact on the actomyosin ATPase activity and nucleotide binding and release. These results show that insert-1 has a critical role in slowing ADP-release and ATP-induced actomyosin dissociation (see data in Supplementary Table S1). Interestingly, the myosin VI structure reveals that the presence of insert-1 does not alter the conformation of switch I relative to the U50kDa subdomain, which is the same in all myosin states visualized to date (Fig. 2). In contrast, the small loop (G304-D313) that follows insert-1 is drastically re-positioned and strongly interacts with Switch I. This loop also protrudes within the nucleotide-binding pocket – resulting in reduction of nucleotide accessibility (Fig. 2). Modeling studies based on the myosin V structure show that when insert-1 is removed, this loop can adopt a conformation close to that adopted by equivalent residues in other myosins, reducing its interactions with Switch I (Fig. 2b). Restrictions in nucleotide binding pocket accessibility explain in large part why the ATP affinity ($1/K_1'$) of actomyosin VI is weak (25mM). Insert-1 likely also influences mobility of switch I relative to the rest of the U50kDa subdomain, which would explain its influence on both ADP-release and ATP-induced dissociation of the motor from actin at saturating ATP concentrations (k_{+2}'). (See supplementary data.)

The role of insert-2 in redirecting the lever arm

Functional studies of chimeric molecules between myosin V and VI were interpreted as evidence that the motor domain, rather than insert-2, provides the major determinants for directionality reversal¹⁵. The nucleotide-free myosin VI structure shows, however, that its motor domain is very similar to that of myosin V – including in the position of the converter, the specific subdomain designed to direct lever arm movement in myosin motors. The converter position is controlled by the specific conformation of two connectors – the relay and the SH1 helix (Fig. 1, 3a,b). Importantly, as found in all plus-end myosins, the set of hydrophobic interactions that maintain the relay and the converter closely linked to one another (in all states of the motor) are also conserved in myosin VI. However, there are sequence variations specific for myosin VI which are clustered in the interface between these two connectors (Supplementary Movie S3). Their effect in modulating the precise orientation of the converter is minimal in the nucleotide-free state of myosin VI, but they could play a critical role in other states of the cycle – such as the pre-powerstroke state.

The truly unique and novel feature of the two myosin VI structures is the reversal of the lever arm direction by insert-2 (P774-Y812 ; Fig. 3). The proximal part of the insert (P774-W787) wraps around the converter, rather than emerging as a straight helix from the converter and the

distal part of the insert (W787-Y812) forms a previously unseen calmodulin-binding motif (see Fig. 4). Both the insert and its associated 4Ca^{2+} -calmodulin make specific interactions with the converter (many involving a variable loop (K719-P731) ; Fig. 3a). The net result of these interactions is that the IQ helix emerges approximately 120° from the position that the IQ helix would in any other myosin (Fig. 3b). This redirects the IQ helix and its bound calmodulin (which form a “lever arm”) toward the minus end of the actin filament (Fig. 1).

Unique structural differences of the myosin VI converter are critical for its close interaction with insert-2 and thus the re-orientation of the lever arm. In particular, the orientation of the last helix of the myosin VI converter differs by about 19° from that found in plus-end myosins when the converters are superimposed (Fig. 3b). This helix is well anchored against the rest of the converter *via* several large hydrophobic side chains. It ends with a proline (P774) which favors a 90° -turn at the beginning of insert-2, that promotes its wrapping around the converter. The variable loop of the converter and the helix that follows create a small hydrophobic cavity in which small hydrophobic side chains of insert-2 fit (Fig. 3c). A break in the helix of insert-2 at position V784 further extends the surface of interaction with the converter (*via* W787 and L788) and also allows the calmodulin to be held by the distal part of this insert to interact with the converter – in particular *via* six salt bridges (Fig. 3d). In contrast, the helix remains straight between insert-2 and the IQ motif and the stiffness at the MD/lever arm junction in this myosin likely is similar to that found for other myosins. The functional converter of myosin VI that redirects the lever arm in the opposite direction in the rigor state thus is composed of the normal converter plus the insert-2/calmodulin.

Insert-2 constitutes a novel calmodulin binding motif

Although the sequence of the myosin VI-specific insert-2 does not correspond to any calmodulin (CaM) binding motif described so far²⁴, we previously reported that it recruits a 4Ca^{2+} -CaM and that dissociation of these Ca^{2+} ions cannot occur and regulate this interaction under physiological conditions¹³. Consistent with these observations, the myosin VI structure reveals that the distal part of insert-2 contains a novel CaM binding motif (Fig. 4). Following the precedent for other targets of CaM²⁵, such as the 1-8-14 motif found in the smooth myosin light chain kinase (MLCK), we name this motif 1-6-14 based on the distance between three key hydrophobic residues (W793, W798, L806) that are buried in the interaction with CaM. Compared to other Ca^{2+} -CaM/target (1-8-14) complexes²⁵, the polarity of this Ca^{2+} -CaM/insert-2 complex is similar with the C-lobe of CaM gripping the N-terminal region of the target sequence (Fig. 4a). While the C-lobe of CaM adopts a classic open conformation, that found for the N-lobe differs from any other complex described so far in being more closed by $\sim 20^\circ$ (Fig. 4b). (It is in fact close to that recently described as a contracted-open conformation²⁶). The target helix thus is found in a much less buried position within this lobe than in a classic open lobe (Fig. 4b). After close inspection of this structure, the selection of such a conformation for the N-lobe seems to be induced by the target motif itself, not the surface contacts this lobe makes with the rest of the motor domain. In particular, the presence of a large hydrophobic side chain in the 6th position, W798, plays a critical role since it interacts strongly with helix A and the last helix of the C-lobe (Fig. 4b). By providing strong hydrophobic interactions and by selecting for relative orientations of the two lobes of CaM that allow them both to interact with the converter, this motif is remarkably well suited to impose a structural role for the recruited CaM (Fig. 4c).

DISCUSSION

What is at first striking about myosin VI is that reverse directionality has been accomplished with only minor changes within the motor domain itself. The first unique insert, insert-1, is not positioned to impact directionality by altering the structure of the motor domain. However, it

does have a major impact on kinetic properties by slowing ADP release and creating a long-lived rigor state. Insert-1 is thus essential for the processivity of the motor and would be critical for the anchoring role of myosin VI, which is dependent on slow ATP binding relative to ADP binding²⁷.

The second unique insert, insert-2, is what initially called attention to myosin VI² and indeed is the most interesting feature of this structure. It reveals that myosin VI has tightly coupled both this insert and a calmodulin to the conventional myosin converter to create a unique converter subdomain. The purpose of this design is to redirect the lever arm toward the minus-end of the actin filament. This undoubtedly is essential for reverse-directionality. For a single-headed myosin, it would reposition the lever arm toward the minus-end of the actin filament in the rigor state (end of powerstroke), and for a two-headed processive myosin, it additionally would bias the unbound lead head toward minus-end-directed binding sites.

The work of Tsiavaliaris *et al.*²⁸ demonstrated that repositioning the lever arm by 180°, rather than 120° as for myosin VI, is sufficient to reverse the directionality using a single-headed plus-end myosin motor domain. To assess if the repositioning of the lever arm is sufficient to explain the myosin VI stroke, a model for myosin VI at the beginning of the powerstroke was obtained using the pre-powerstroke state of plus-end motors (see Methods). A minus-end directed movement (i.e. reversal) is indeed produced but the component of the displacement parallel to the actin filament would only be ~2.5 nm (Fig. 5a). This is five times less than the 12 nm stroke size measured from optical trap studies for a MD^{ins2}IQ molecule¹⁴. A similar modeling for a truncated myosin V motor would predict a stroke of ~7 nm (Fig. 5b), which is what was measured in optical trap studies⁸. Note that for either myosin V or this myosin VI model, the rotation of the converter contributes a plus-end directed stroke. While this contribution adds to the lever arm displacement for a plus-end directed motor, it must be overcome by the lever arm contribution (for example, by increasing lever arm length) for a minus-end directed motor. This is illustrated in a model of the artificial lever arm, which achieved reversal of myosin I²⁸ using a plus-end converter rotation and a 14 nm long lever arm (Fig. 5c).

Since a MD^{ins2}IQ myosin VI molecule has an inexplicably large¹⁴ minus-end directed stroke² with a very short (4 nm) lever arm, we must conclude that myosin VI has a quite different pre-powerstroke structure from other myosins. This could be accomplished in at least two fundamentally different ways. First, the position of the converter could differ from that of other characterized myosins in all of the nucleotide states of myosin VI. There is already evidence from cryo-EM that the rotation from the actomyosin ADP (A.M.ADP) to the rigor state is in a different direction². Since the rearrangements necessary for this transition are of small amplitude, it is possible to imagine that they differ in myosin VI due to the small alterations in the nature of the β -sheet and SH1 helix (Supplementary movies S2 and S3). But major rearrangements occur upon ATP-binding to generate the post-rigor and pre-powerstroke states¹⁸ and it is difficult to imagine how these subtle motor domain differences could cause the nearly 90° change in rotation of the converter in the pre-powerstroke state that would be necessary to generate the large step size of the MD^{ins2}IQ molecule¹⁴ (see Fig. 5d). Such an altered rotation would likely require a major redesign of the motor domain, which the structure of myosin VI clearly reveals did not occur.

The second and more easily envisioned possibility is that myosin VI has not truly reversed the normal myosin powerstroke, but has essentially abolished it and evolved a mechanism similar to that proposed for “conventional” kinesin^{29,30}. In kinesin, it is thought that movement is accomplished by a reversible, nucleotide-state dependent docking and undocking of its neck-linker region. In the two-headed kinesin molecule, intra-molecular strain gates the docking and undocking, while the docked head biases the diffusive search for a new tubulin binding site of

the unbound head during processive movement. To apply this mechanism to myosin VI, either the insert-2/CaM or the entire converter subdomain must uncouple from the motor domain in the pre-powerstroke state. In the case of insert-2/CaM, it appears to be an integral part of the converter, and as can be seen in the pre-powerstroke model (Fig. 5a), there would be no steric hindrance between this unique myosin VI converter and the motor domain to drive such an uncoupling. However, we propose that uncoupling of the entire converter from the motor domain could be induced by an SH1 helix unwinding in the post-rigor and pre-powerstroke states (Fig. 5e). A cluster of sequence differences specific for myosin VI are indeed found in the cavity of the SH1 helix (Supplementary movie S3) and there is structural evidence for unwinding of this connector in myosin II following nucleotide binding³¹. Thus in myosin VI, the SH1-helix would be roughly analogous to the neck-linker of kinesin.

Consistent with this hypothesis is data that placed fluorescent probes either on the IQ-CaM or on the N-terminal subdomain of two-headed myosin VI³². Using FIONA (fluorescence imaging with one nanometer accuracy), large fluctuations in the position of the IQ-CaM, but not in the position of the motor domain, were observed during processive movement. These fluctuations disappeared when ATP was removed and the heads were strongly bound to actin with either ADP or no nucleotide³². Thus, based on these observations and our new structural insights, we hypothesize that myosin VI (M.ADP.P_i) binds to actin with an uncoupled converter domain. In the absence of strain, strong binding to actin accompanies cleft closure via the central β -sheet distortion. These rearrangements would lead to an interface between the SH1 helix and the N-terminal subdomain that would favor recoupling of the converter to the motor domain in the strong actin-binding A.M.ADP state. Recoupling of the lead head for a two-headed processive myosin would be prevented, or greatly slowed, until the rear head detaches. This is consistent with both the FIONA study³² and direct measurements of pyrene-actin quenching (strong binding) with the myosin VI dimer in the presence of ATP³³. When ADP dissociates, further cleft closure would lead to a small rotation of the lever arm in the minus-end direction². The net displacement on actin (stroke) would be the difference in the average position of the lever arm when uncoupled and the stable rigor position.

This uncoupling model could account for ~6–7 nm movement of the MD^{ins2}IQ construct, assuming that there is no positional bias of the uncoupled converter (Fig. 5e). However, as depicted in this model, any biasing toward the plus end of the actin filament would increase the stroke and step size. Such biasing might come from the relay, which based on the myosin II structure with an unwound SH1 helix³¹, would maintain connections with the converter and would be plus-end directed due to the loss of the steric hindrance that normally bends the relay helix in the minus-end direction in the pre-powerstroke state^{7,31,34}. Additional experiments and structures will be necessary to test this proposed mechanism of directionality reversal. Undoubtedly, this unique myosin family member has yet more surprises to reveal.

METHODS

Crystallization and Data collection

Protein expression and purification have been previously described^{2,13} and are detailed in Supplementary Methods. Crystals of myosin VI were first obtained with the short MD^{ins2}IQ [1-839] construct by vapor diffusion. Spontaneous nucleation occurred at 4°C in hanging drops using equal amounts of reservoir solution (containing 8–10% PEG 8000, 50mM Tris pH 8.0, 3% iso-propanol, 3% tert-butanol) and stock solution of the Myosin VI MD^{ins2}IQ at 10–12 mg/mL. Crystals of the MD^{ins2} [2-816] and long MD^{ins2}IQ [1-859] constructs were initially obtained by cross-seeding with crystals of the short MD^{ins2}IQ and then improved by seeding in solution containing 8–10% PEG 8000, 50mM MES pH 6.7, 150mM NH₄.SO₄, 3% iso-propanol, 3% tert-butanol. Prior to freezing and data collection, the crystals were transferred stepwise into a final cryoprotectant solution containing 16% PEG 8000 with 25% glycerol. X-

ray data sets were collected at the European Synchrotron Radiation Facility beamlines at 100 K. The MD^{ins2} construct was crystallized in a monoclinic crystal form, while both MD^{ins2}IQ constructs were crystallized in an orthorhombic one. The long MD^{ins2}IQ crystals are similar to those of the short MD^{ins2}IQ except for the b-axis which length accommodates for the difference in lever arm length. The short MD^{ins2}IQ, long MD^{ins2}IQ and MD^{ins2} crystals diffracted to 3.5 Å, 2.9 Å and 2.4 Å resolution, respectively. All datasets were integrated and scaled with either the HKL package³⁵ or the CCP4 suite³⁶ programs (see Table 1 and Supplementary Table S2 for statistics on the data collection).

Structure determination and refinement

The myosin VI structure was solved by molecular replacement using the myosin V MDE model (PDB code 1OE9) with the program AMoRe³⁷ using data at 3.5 Å resolution from the short MD^{ins2}IQ or the MD^{ins2} crystals. Several steps of rigid body fitting were performed with AMoRe (each subdomain has been considered as a rigid group). Model building and refinement of the motor domain, the insert-2 and its 4Ca²⁺-bound CaM was carried out at 2.4 Å resolution using data from the MD^{ins2} crystals with Turbo³⁸, and CNS1.1³⁹ and Refmac5 (CCP4 suite)³⁶. The long MD^{ins2}IQ structure was solved by molecular replacement³⁷ using the coordinates from this 2.4 Å refined myosin VI MD^{ins2} structure. Clear density was easily interpreted for the N-terminal part of the IQ helix and most of the C-terminal lobe of its CaM, which were modeled as poly-alanines. The C-terminal part of the IQ motif and most of the N-lobe of its CaM are not stabilized by crystal packing interactions. They have a high level of flexibility in the crystal and were not included in the refined model (See Supplementary Fig. S1). Water molecules have been placed with Arp/Warp program⁴⁰. Crystallographic statistics are summarized in Table 1. Note that all diagrams for the figures and the movies were computed using MOLSCRIPT/Raster3D⁴¹.

Pre-powerstroke state modeling

Models of the myosin VI and V pre-powerstroke states were obtained using known myosin II pre-powerstroke structures. The scallop striated muscle myosin II pre-powerstroke structure⁴² (1DFL) provided coordinates for the motor domain. Position for the myosin VI and V converter/lever arms were obtained by superimposing their converter with that of the myosin II converter. Similarly, the lever arm for the engineered reverse myosin I motor was obtained from a molecular model³¹ (D. Manstein, personal communication). Note that this modeled myosin VI pre-powerstroke state assumes that the converter and the insert-2/CaM interactions are not broken during the catalytic cycle, which seems reasonable in view of their strong interactions. Note that no steric clash is generated between the insert-2/CaM and the motor domain when this myosin II converter rotation is applied to myosin VI. The pre-powerstroke and nucleotide-free (1OE9, 2BKI) structural models were docked on F-actin as previously described¹⁸ and a schematic drawing is shown for clarity in Fig. 5. The stroke resulting from this model was measured as the component of the displacement parallel to the actin filament.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a grant from the National Institutes of Health (AR-048931) to H.L.S and A.H., grants from the CNRS and the ARC to A.H., as well as predoctoral fellowships from the Quebec government and the FRM to A.B. We thank the staff of the European Synchrotron Radiation Facility for assistance during data collection. We are also grateful to Anna Li and Damien Garbett for technical assistance in preparing the recombinant proteins and to Jérôme Cicolari for assistance in crystallization experiments.

References

1. Berg JS, Powell BC, Cheney RE. A millennial myosin census. *Mol Biol Cell* 2001;12:780–794. [PubMed: 11294886]
2. Wells AL, Lin AW, Chen LQ, Safer D, Cain SM, Hasson T, Carragher BO, Milligan RA, Sweeney HL. Myosin VI is an actin-based motor that moves backwards. *Nature* 1999;401:505–508. [PubMed: 10519557]
3. Geisbrecht ER, Montell DJ. Myosin VI is required for E-cadherin-mediated border cell migration. *Nat Cell Biol* 2002;4:616–620. [PubMed: 12134162]
4. Hasson T. Myosin-VI: two distinct roles in endocytosis. *J Cell Sci* 2003;116:3453–3461. [PubMed: 12893809]
5. Buss F, Spudich G, Kendrick-Jones J. Myosin VI: Cellular functions and motor properties. *Annu Rev Cell Dev Biol* 2004;20:649–676. [PubMed: 15473855]
6. Millo H, Leaper K, Lazou V, Bownes M. Myosin VI plays a role in cell-cell adhesion during epithelial morphogenesis. *Mech Dev* 2004;121:1335–1351. [PubMed: 15454264]
7. Geeves MA, Holmes KC. Structural mechanism of muscle contraction. *Annu Rev Biochem* 1999;68:687–728. [PubMed: 10872464]
8. Purcell TJ, Morris C, Spudich JA, Sweeney HL. Role of the lever arm in the processive stepping of myosin V. *Proc Natl Acad Sci USA* 2002;99:14159–14164. [PubMed: 12386339]
9. Sakamoto T, Wang F, Schmitz S, Xu Y, Molloy JE, Veigel C, Sellers JR. Neck length and processivity of myosin V. *J Biol Chem* 2003;278:29201–29207. [PubMed: 12740393]
10. Ruff C, Furch M, Brenner B, Manstein DJ, Meyhofer E. Single-molecule tracking of myosins with genetically engineered amplifier domains. *Nature Struct Biol* 2001;8:226–229. [PubMed: 11224566]
11. Rock RS, Rice SE, Wells AL, Purcell TJ, Spudich JA, Sweeney HL. Myosin VI is a processive, backwards motor with a large step size. *Proc Natl Acad Sci USA* 2001;98:13655–13659. [PubMed: 11707568]
12. Nishikawa S, Homma K, Komori Y, Iwaki M, Wazawa T, Hikikoshi Iwane A, Saito J, Ikebe R, Katayama E, Yanagida T, Ikebe M. Class VI myosin moves processively along actin filaments backward with large steps. *Biochem Biophys Res Commun* 2002;290:311–317. [PubMed: 11779171]
13. Bahloul A, Chevreux G, Wells AL, Martin D, Nolt J, Yang Zhaohui, Chen L-Q, Potier N, Dorsselaer AV, Rosenfeld SS, Houdusse A, Sweeney HL. The unique insert in myosin VI is a structural calcium-calmodulin binding site. *Proc Natl Acad Sci USA* 2004;101:4787–4792. [PubMed: 15037754]
14. Rock RS, Ramamurthy B, Dunn AR, Beccafico S, Rami BR, Morris C, Spink BJ, Franzini-Armstrong C, Spudich JA, Sweeney HL. A flexible domain is essential for the large step size and processivity of myosin VI. *Molecular Cell* 2005;17:603–9. [PubMed: 15721263]
15. Homma K, Yoshimura M, Saito J, Ikebe R, Ikebe M. The core of the motor domain determines the direction of myosin movement. *Nature* 2001;412:831–834. [PubMed: 11518969]
16. Coureux P-D, Wells AL, Ménétrety J, Yengo CM, Morris CA, Sweeney HL, Houdusse A. A structural state of the myosin V motor without bound nucleotide. *Nature* 2003;425:419–423. [PubMed: 14508494]
17. Reubold TF, Eschenburg S, Becker A, Kull FJ, Manstein DJ. A structural model for actin-induced nucleotide release in myosin. *Nature Struct Biol* 2003;10:826–830. [PubMed: 14502270]
18. Coureux P-D, Sweeney HL, Houdusse A. Three myosin V structures delineate essential features of chemo-mechanical transduction. *EMBO J* 2004;23:4527–4537. [PubMed: 15510214]
19. Holmes KC, Schröder RR, Sweeney HL, Houdusse A. The structure of the rigor complex and its implications for the power stroke. *Philos Trans R Soc Lond B Biol Sci* 2004;359:1819–28. [PubMed: 15647158]
20. De La Cruz EM, Ostap EM, Sweeney HL. Kinetic mechanism and regulation of myosin VI. *J Biol Chem* 2001;276:32373–32381. [PubMed: 11423557]
21. De La Cruz EM, Wells AL, Rosenfeld SS, Ostap EM, Sweeney HL. The kinetic mechanism of myosin V. *Proc Natl Acad Sci USA* 1999;96:13726–31. [PubMed: 10570140]
22. Sweeney HL, Houdusse A. The motor mechanism of myosin V: insights for muscle contraction. *Philos Trans R Soc Lond B Biol Sci* 2004;359:1829–1842. [PubMed: 15647159]

23. Rosenfeld SS, Houdusse A, Sweeney HL. Magnesium regulates ADP dissociation from myosin V. *J Biol Chem* 2005;280:6072–6079. [PubMed: 15579901]
24. Rhoads, AR.; Friedberg, F. Sequence motifs for calmodulin recognition; FASEB J. 1997. p. 331-340.<http://calcium.uhnres.utoronto.ca/ctdb/ctdb/home.html>: Calmodulin Target Database.
25. Meador WE, Means AR, Quirocho FA. Target enzyme recognition by calmodulin: 2.4 Å structure of a calmodulin-peptide complex. *Science* 1992;257:1251–1255. [PubMed: 1519061]
26. Fallon JL, Quirocho FA. A closed compact structure of native Ca(2+)-calmodulin. *Structure* 2003;11:1303–1307. [PubMed: 14527397]
27. Altman D, Sweeney HL, Spudich JA. The mechanism of myosin VI translocation and its load-induced anchoring. *Cell* 2004;116:737–749. [PubMed: 15006355]
28. Tsiavaliaris G, Fujita-Becker S, Manstein DJ. Molecular engineering of a backwards-moving myosin motor. *Nature* 2004;427:558–561. [PubMed: 14765199]
29. Rice S, Lin AW, Safer D, Hart CL, Naber N, Carragher BO, Cain SM, Pechatnikova E, Wilson-Kubalek EM, Whittaker M, Pate E, Cooke R, Taylor EW, Milligan RA, Vale RD. A structural change in the kinesin motor protein that drives motility. *Nature* 1999;402:778–784. [PubMed: 10617199]
30. Rosenfeld SS, Fordyce PM, Jefferson GM, King PH, Block SM. Stepping and stretching: how kinesin uses internal strain to walk processively. *J Biol Chem* 2003;278:18530–18536.
31. Houdusse A, Kalabokis VN, Himmel D, Szent-Gyorgyi AG, Cohen C. Atomic structure of scallop myosin subfragment S1 complexed with MgADP: a novel conformation of the myosin head. *Cell* 1999;97:459–470. [PubMed: 10338210]
32. Yildiz A, Park H, Safer D, Yang Z, Chen LQ, Selvin PR, Sweeney HL. Myosin VI steps via a hand-over-hand mechanism with its lever arm undergoing fluctuations when attached to actin. *J Biol Chem* 2004;279:37223–37226. [PubMed: 15254036]
33. Robblee JP, Olivares AO, De la Cruz EM. Mechanism of nucleotide binding to actomyosin VI: evidence for allosteric head-head communication. *J Biol Chem* 2004;279:38608–38617. [PubMed: 15247304]
34. Sasaki N, Ohkura R, Sutoh K. *Dictyostelium* Myosin II mutations that uncouple the converter swing and ATP hydrolysis cycle. *Biochemistry* 2003;42:90–95. [PubMed: 12515542]
35. Otwinowski Z, Minor W. Processing X-ray diffraction data collected in oscillation mode. *Methods Enzymol* 1997;276:307–326.
36. Collaborative Computational Project No. 4. The CCP4 suite: programs for protein crystallography. *Acta Crystallogr D* 1994;50:760–763. [PubMed: 15299374]
37. Navaza J. AMoRe: an automated package for molecular replacement. *Acta Crystallogr A* 1994;50:157–163.
38. Roussel, A.; Cambillaud, C. Silicon Graphics Geometry Partner Directory. Silicon Graphics; Mountain View, CA: 1989. p. 77-78.
39. Brünger AT, et al. Crystallography & NMR system: a new software suite for macromolecular structure determination. *Acta Crystallogr D* 1998;54:905–921. [PubMed: 9757107]
40. Perrakis A, Morris RM, Lamzin VS. Automated protein model building combined with iterative structure refinement. *Nature Struct Biol* 1999;6:458–463. [PubMed: 10331874]
41. Kraulis PJ. MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J Appl Crystallogr* 1991;24:946–950.
42. Houdusse A, Szent-Györgyi A, Cohen C. Three conformational states of scallop myosin subfragment S1. *Proc Natl Acad Sci USA* 2000;97:11238–11243. [PubMed: 11016966]

Figure 1.

Crystal structure of nucleotide-free myosin VI. **a**, A ribbon diagram representation of the myosin VI structure highlights its two specific inserts, the four subdomains (N-terminal, U50kDa, L50kDa and Converter) of the motor domain and two connectors (relay and SH1 helix) that control the converter. Insert-2 recruits a 4Ca^{2+} -calmodulin while the IQ motif interacts with an apo-calmodulin. **b**, Comparison with the myosin V rigor-like state¹⁶. Note the similarity in the overall conformation of the motor domains and the striking difference in the orientation of the lever arm (composed of an IQ motif and associated light chain). Displayed on the right are the same structures rotated by 180° along the actin filament axis (black arrow), obtained by fitting the EM electron density map of the actin-myosin VI rigor complex².

Figure 2.

Insert-1 modulates nucleotide binding and Switch I flexibility. **a**, Insert-1 is found at the surface of the U50kDa subdomain near the nucleotide-binding site. **b**, The same region is shown for myosin V which lacks this insert. Note the conformational change in the loop (yellow) in which insert-1 is inserted. Residues that interact with Switch I are shown in ball-and-sticks and ATP molecules are modeled in white ball-and-sticks. Also, on the upper-right corner, a surface CPK representation of this region is depicted in a different orientation in order to evaluate the accessibility of the nucleotide-binding pocket for the ATP molecule. **c**, Sequence alignment of Myosin V and VI near insert 1 colored as in a, b. Residues involved in interaction with Switch I are underlined.

Figure 3.

Reorientation of the myosin VI lever arm by its unique converter. **a**, The proximal part of insert-2 (P774-W787 ; purple/grey) interacts on the surface of the converter, in particular with a loop whose sequence is highly variable among myosin classes, but quite conserved within the myosin VI class. The distal part of insert2 (W787-Y812 ; purple/dark-pink) recruits a 4Ca²⁺-calmodulin. **b**, Compared to myosin V (black), the linkages found between the relay/SH1 helix connectors and the converter β -sheet are conserved but the orientation of the last helix of the converter (G761-D773) differs by 19°. Note that W702 of myosin V (A709 in myosin VI) would interfere with this orientation. **c**, Close up view of the apolar interactions that stabilize the proximal part of insert-2 on the surface of the converter. Note also how three anchoring hydrophobic side chains (light red) stabilize the orientation of the last helix. **d**, Close up view of the salt bridge interactions between the converter and both lobes of calmodulin.

Figure 4.

A new calmodulin binding motif that interacts strongly with 4Ca^{2+} -CaM. **a**, The overall conformation and polarity of the insert-2/CaM complex (new 1-6-14 motif) is compared to those observed when CaM interacts with myosin light chain kinase (MLCK) (classic 1-8-14 motif) (target peptides superimposed). Note that in both cases, the C-lobe of CaM in an open conformation grips the N-terminal region of the target sequence, largely via the first anchoring hydrophobic residue (**W793/W800**). **b**, In contrast, comparison of the N-lobes (helices A and D superimposed) shows differences in their conformation (closure differs by 20°) and in the target peptide position (note the 14th anchoring residue position) within the lobe. Note that the 6th anchoring residue of the 1-6-14 motif (**W798**) interacts strongly with both lobes of CaM

(helices A and H). **c**, Sequence comparison of the two CaM-binding motifs. The letter n, c or b indicates whether each residue of these motifs interact with either the N-, the C- or both lobes of CaM, respectively.

Figure 5.

Directionality of movement and power stroke in myosin motors. **a,b**, Schematic drawings of the myosin V and VI structural models (see Methods) prior and after force generation (using similar colors as in Fig. 1). Note in particular how conformational changes in the relay (yellow) and SH1 helix (red) lead to the rotation (black arrow) of the converter (green). The red arrow represents the predicted F-actin displacement (stroke) for these models while the green arrow indicates the converter contribution for this stroke. **(c)** The solid arrow indicates the stroke that would be produced with a lever arm of ~ 4 nm (that equivalent to one IQ motif), the dotted arrow corresponds to the stroke generated by a ~14 nm lever arm as described for this engineered motor³¹. **d, e**, Two mechanisms could account for the ~12 nm stroke of the myosin

VI MD^{ins2}IQ. If the converter remains coupled to the motor domain (**d**), it must adopt an orientation that differs by $\sim 90^\circ$ from that found for plus-end motors in the pre-powerstroke state. (**e**) Alternatively, unwinding of the SH1 helix in the weak actin-binding states would decouple the converter from the motor domain. In this case, the relay/converter interactions would be maintained, but the relay helix would not be bent in the pre-powerstroke state since steric clashes with the SH1 helix are eliminated. Thus the converter would be biased toward the plus-end of the actin filament. Re-coupling of the converter to the motor domain would occur upon strong binding to actin.

Table 1
Statistics on Data Collection and Refinement

X-ray Stats		
	Long MD^{ins2}1Q	MD^{ins2}
Refinement		
Resolution (Å)	2.9	2.4
$R_{\text{work}}/R_{\text{free}}$	26.3/30.3	20.6/25.4
No. atoms		
Protein	7991	7403
Ligand/ion	5/4	36/4
Water	49	285
B-factors		
Protein	46.01	48.76
Ligand/ion	35.2/79.4	60.9/79.7
Water	28.37	44.90
R.m.s deviations		
Bond lengths (Å)	0.013	0.011
Bond angles (°)	1.314	1.326