Published in final edited form as:

J Mol Biol. 2011 August 5; 411(1): 16–26. doi:10.1016/j.jmb.2011.05.019.

EPR SPECTRA AND MOLECULAR DYNAMICS AGREE THAT THE NUCLEOTIDE POCKET OF MYOSIN V IS CLOSED AND THAT IT OPENS ON BINDING ACTIN

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Abstract

We have used EPR spectroscopy and computational modeling of nucleotide-analog spin probes to investigate conformational changes at the nucleotide site of myosin V (MV). We find that in the absence of actin, the mobility of a spin-labeled diphosphate analog (SLADP) bound at the active site is strongly hindered, suggesting a closed nucleotide pocket. The mobility of the analog increases when the MV•SLADP complex binds to actin (A), implying an opening of the active site in the A•MV•SLADP complex. The probe mobilities are similar to those seen with myosin II, despite the fact that myosin V has dramatically altered kinetics. Molecular dynamics simulation was used to understand the EPR spectra in terms of the X-ray database. The X-ray structure of MV•ADP•BeFx shows a closed nucleotide site and has been proposed to be the detached state. The MV•ADP structure shows an open nucleotide site and has been proposed to be the A•MV•ADP state at the end of the working powerstroke. Molecular dynamics simulation of SLADP docked in the closed conformation gave a probe mobility comparable to that seen in EPR spectra of the MV•SLADP complex. The simulation of the open conformation gave a probe mobility that was 35°–40° greater than that observed experimentally for the A•MV•SLADP state. Thus EPR, X-ray diffraction and computational analysis support the closed conformation as a myosin V state that is detached from actin. The MD results indicate that the MV•ADP crystal structure is super-opened, which may correspond to the strained actin-bound post-powerstroke conformation resulting from head-head interaction in the dimeric, processive motor.

Keywords

myosin V; electron paramagnetic resonance spectroscopy; spin probes; Switch 1; molecular dynamics simulation

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INTRODUCTION

Myosin V is a motor protein that transports intracellular vesicles within the cell¹⁻⁵ moving processively along actin filaments. In contrast to myosin II, which exhibits a low duty-cycle with myosin detached from actin during the majority of the ATPase cycle, processivity requires a high duty-cycle to prevent the myosin-organelle complex from detaching prematurely from the actin roadway. Kinetic analysis of isolated myosin V in solution indicates that the rate of ADP release from the A•MV•ADP complex (A=actin, MV=myosin V) is slow, while under physiological conditions the rates of ATP binding and dissociation of the A•MV complex, ATP hydrolysis in the MV•ATP state, and Pi release from the A•MV•ADP•Pi state are all much more rapid⁶⁻⁹. Thus in contrast to myosin II, the predominant state in active sliding becomes the strongly bound A•MV•ADP state. This rate-limiting, strongly bound state allows for processive motion along the actin filament.

The original x-ray structures of myosin showed the nucleotide phosphates tightly enclosed in a “phosphate tube” composed of the P-loop, Switch 1 and Switch 2 motifs¹⁰⁻¹⁴, so named due to their homology with the corresponding motifs in the more well studied G-proteins. The observation that analogs containing moieties that were too large to fit through the closed phosphate tube still bound to myosin demonstrated that the closed phosphate tube must open at some point in the kinetic cycle¹⁵. X-ray structures were subsequently solved for a myosin II fusion protein¹⁶, myosin V¹⁷ and myosin VI¹⁸ that showed an open nucleotide-binding site. Myosin is an α - β - α protein with α -helices flanking both sides of a core β -sheet platform. The X-ray structures show an open phosphate tube resulting from a displacement of an otherwise invariant Switch 1 domain away from the nucleotide triphosphates, resulting from a flexing of the core β -sheet. Furthermore, the 50 kDa cleft is closed in the X-ray structure. This is in contrast to the opening of the nucleotide site in kinesin-family motors. Here the core β -sheet is basically invariant and the opening of the nucleotide site results from Switch 1 undergoing a conformational change from a loop to α -helix-loop motif as it pulls away from the nucleotide phosphates, opening the nucleotide site. The open MV•ADP structure has been proposed to be an actin-bound state at the end of the working powerstroke¹⁷. Electron microscopy¹⁹ likewise suggested that the opening of the nucleotide pocket was associated with the strongly bound A•M•D state in myosin II. A closed 50 kDa cleft along with an open nucleotide site would promote tight binding to actin and facilitate nucleotide release at the end of the powerstroke. Spectroscopic approaches^{16,20-23} have likewise implied that the myosin nucleotide pocket can open. In particular, EPR spectroscopy has indicated that for myosin II, the opening of the nucleotide pocket is not an all-or-none event^{21,22}. When detached from actin, the nucleotide pocket is closed. When myosin II binds to actin in the strongly bound A•M•ADP state, EPR probe mobility shows that an open conformation is now present that is in equilibrium with a closed conformation. The equilibrium between the open and closed conformations is temperature dependent, with the open conformation favored by more physiological temperatures.

As noted, myosin V has perturbed kinetics and energetics for the strongly bound A•MV•ADP state relative to those observed for myosin II. Thus we have used EPR spectroscopy to investigate the conformation of the nucleotide pocket as a function of nucleotide state and actin binding. We find that despite the perturbed kinetics, only the closed conformation is present when myosin V is detached from actin, irrespective of the nucleotide at the active site. In the strongly bound A•MV•ADP state, there is again an equilibrium between open and closed conformations of the nucleotide site. However, the equilibrium favors the open state more than is the case for myosin II. Molecular dynamics simulations are used to compare probe mobility experimentally observed for the closed and open conformations of the nucleotide site with that expected from the X-ray structures with open and closed nucleotide-binding sites. The computational results indicate that the

mobility experimentally observed for the MV•ADP state is compatible with the myosin V X-ray structure with a closed nucleotide-binding site. This structure has been proposed to be the post-rigor state immediately following ATP binding at the end of the powerstroke. The MV•ADP X-ray structure with an open conformation of the myosin V nucleotide has been postulated to be a strongly bound A•MV•ADP state²⁴. However, the mobility experimentally observed for the strongly bound A•MV•SLADP state is significantly less (35°-40°) than the computationally modeled mobility to be expected from this conformation of the nucleotide pocket. This result is not compatible an assignment for the MV•ADP X-ray state as a strongly bound A•MV•ADP state at the end of the powerstroke.

RESULTS

Fig. 1 shows the spectra obtained from myosin V in the absence of actin. The truncated central peaks P2 and P4 are from unbound spin probe tumbling freely in solution. When the spin probe binds at the nucleotide site, the adjacent protein surface restricts the motion of the probe due to the physical limitation on the region of space it can explore in the face of thermal agitation. This results in a broadening of the spectrum. The low-field peak, P1, and the high-field dip, P5, are from bound nucleotide. The central peak, P3, arises from both bound nucleotide and nucleotide that is free in solution. The top spectrum (blue) in Fig. 1 is from the MV•2'-SLADP state at 23°C. Griffith and Jost²⁵ have shown that the magnitude of the P1-P5 splitting can be related to rapid, uniform probe motion within a cone of revolution of defined vertex angle. The greater the splitting between the P1 and P5 components of a spectrum, the more restrictive is the adjacent protein surface and the smaller is the vertex angle of the related cone of revolution. Although the restricting protein surface is most certainly not a cone of revolution, the analysis serves as a useful approximation to relate the magnetic field splitting to a physical model. In Fig. 1 the P1-P5 splitting for the spectrum from the MV•2'-SLADP state is 5.9mT, corresponding to a cone angle of 81.7°. Thus the spin probe is reporting a relatively tightly closed nucleotide site. All spectra in Fig. 1 were accumulated at 23°C. The spectrum of the MV•2'-SLADP state at 2°C shows a slight decrease in splitting (0.5mT) implying a slightly more closed nucleotide pocket at the lower temperature (data not shown). The spectral components from unbound probe (P2-P4) are truncated in Fig. 1 to enhance display of the bound components (P1, P5). A full spectrum without truncation is shown in Fig. S1.

We examined the conformation of the nucleotide pocket in a number of different nucleotide states. Magnesium was removed by addition EDTA to the buffer. The splitting is similar to that seen for MV•2'-SLADP in the presence of magnesium (cyan). The lower two panels in Fig. 1 show the spectra obtained for the MV•2'-SLADP•BeFx (red) and MV•2'-SLADP•AlFx (black). ADP•BeFx/AlFx are stably trapped analogs of the triphosphate state. The tetrahedral arrangement of the fluorine atoms in BeFx makes 2'-SLADP•BeFx an analog of ATP. The planar arrangement of the fluorine atoms in AlFx makes 2'-SLADP•AlFx an analog of ATP in the hydrolysis transition-state intermediate. The low-field to high-field splitting in the MV•2'-SLADP•BeFx/AlFx states are slightly greater than in the corresponding MV•2'-SLADP state indicating a slightly more closed nucleotide pocket. Despite these small differences in the spectra of Fig. 1, taken *in toto*, the data suggest that the myosin V nucleotide pocket is tightly closed in all nucleotide states in the absence of actin. Low-field to high-field spectral splittings and corresponding cone angles are summarized in Table 1.

Fig. 2 shows spectra from myosin V when bound to actin as a function of temperature. The spectrum from MV•2'-SLADP, 23°C from Fig. 1 is shown in Fig. 1E (blue) for comparison. As is clear from the spectra, the primary P1 and P5 components of the spectra show a decreased splitting in the A•MV•2'-SLADP state compared to the actin-free state. Thus the

EPR probe is reporting an opening of the nucleotide pocket when the MV•2'-SLADP state binds to actin. There is a slight decrease in the P1-P5 splitting in the A•MV•2'-SLADP state as temperature increases. However, comparing the MV•2'-SLADP and A•MV•2'-SLADP spectra at 23°C, the EPR probe indicates a 23° increase in the cone angle approximating the nucleotide pocket when myosin binds to actin (36° increase at 2°C). Importantly, however, the spectra for the A•MV•2'-SLADP state show two bound components that are in equilibrium. This is most easily seen in the spectra at 12°C (Fig. 2C, black) and 2°C (Fig. 2D, green). There is a broad low-field shoulder in the P1 component and a much broader P5 dip. Thus the additional component has a broader splitting than the major component, with P1-P5 splitting similar to that seen in myosin in the absence of actin. Thus when myosin is bound to actin in the diphosphate state, there is an equilibrium between more open and more closed conformations of the nucleotide site. The spectra can be deconvoluted into the respective components as described previously^{26,27}. Letting $K_{eq} = [\text{fraction of myosin showing the more mobile component of the spectrum}]/[\text{fraction showing the more immobilized component}]$, K_{eq} changes from 0.88 at 2°C to 8.60 at 34°C. At the most physiological temperature in Fig. 2, approximately 90% of the myosin in the strongly bound A•MV•2'-SLADP state have nucleotide pockets that are in the more open conformation. The spectral components from unbound probe (P2-P4) are truncated in Fig. 2 to enhance display of the bound components (P1, P5). A full spectrum without truncation is shown in Fig. S1.

Molecular modeling of EPR probe motion

EPR probe motion shows that myosin V has a closed nucleotide site when detached from actin. When myosin V binds to actin in the strongly bound A•MV•ADP state, the nucleotide site opens up. X-ray structures exist for myosin V with the nucleotide site in both open¹⁸ and closed²⁴ conformations. The closed X-ray-structure conformation has been proposed to represent the structure of myosin when detached from actin²⁴. The open conformation has been proposed to be the strongly bound A•MV•ADP state¹⁸. The latter assignment is an extrapolation, however, since actin was not present in the X-ray structure. Figure 3 shows an overlay of the nucleotide site of the two structures with 2'-SLADP (red, gray, magenta) docked at the nucleotide site. In the closed conformation, Switch 1 (magenta) and a surface loop (yellow) are adjacent to the spin moiety (dark gray) restricting motion. In the open conformation, Switch 1 and the surface loop are displaced away from the spin moiety due to a flexing of the β -sheet core of the protein. Due to the 8-10Å displacement of Switch 1 in the open conformation, there would be less steric interference to probe motion and a more mobile probe would be expected, as is observed when myosin binds to actin.

Defining myosin physiology in terms of the X-ray database is a major goal of the field as related to efforts to correlate myosin structure and function. Quantitative comparison is essential for this goal. However, quantitative analysis of the EPR spectra has been previously limited to analyzing the probe mobility as rapid motion in a cone of revolution. Although a useful approximation, the approach has obvious limitations in trying to correlate experimental observations of EPR probe mobility with the structural database. Given an X-ray structure of the protein, a more detailed approach is to use molecular dynamics (MD) simulation to model the motion of the probe in the face of an uneven protein surface^{28,29} and then compare with experiment. That is the approach we take here.

The experimentally observed EPR spectrum is a function of the time-dependent angular deviation, θ , of the EPR probe from a fixed axis in the laboratory reference frame (Fig. S3). The probe executes a probabilistic walk as a function of time (Fig. S4). The MD simulation (Methods) models this walk as a function of time. The aggregate angular distribution of the probe, $\rho(\theta)$, modeled by the simulation can be used to determine an order parameter²⁵. The order parameter can then be compared to that from an experimentally observed EPR

spectrum to determine whether the X-ray structure could yield the experimentally observed spectrum.

Fig. 4 shows representative MD-simulated orientations of 2'-SLADP docked in the nucleotide pocket of myosin V with a closed nucleotide pocket (PDB ID 1W7J, ref ²⁴) during a simulation. The nitroxide bond in the pyrrolidine ring of the EPR probe moiety (Fig. S2) is in purple. Different orientations of the probe result from thermal motion that is further restricted by interaction with the adjacent protein surface (cyan). It is the orientational distribution of the unpaired electron in the nitroxide bond that determines the observed EPR spectrum. Figs. 5A, B give the probe angle, θ , as a function of time over a 14 ns period for 2'-SLADP docked at the nucleotide site of the X-ray structures of the open and closed conformations of myosin V, respectively. As would be anticipated, the angular distribution is more restricted for the structure with the closed nucleotide site. The aggregate orientational distributions, $\rho(\theta)$, for the open and closed simulations are shown in Fig. 6. Order parameters, S_m , for the simulations can be determined from Eqs. 1, 2 (Methods). For the closed X-ray structure, $S_m = 0.62$. For the open structure, $S_m = 0.22$. The equivalent cones angles of mobility that would be observed are 87° and 141° , respectively. Visualization of the structure as a function of time shows that the probe mobility of the open state is not due to excessive mobility of the nucleotide at the active site. The ADP moiety remains where initially docked. The docking was based on the location of ADP at the active site of the X-ray structure.

DISCUSSION

We have used EPR spectroscopy of spin-labeled nucleotides bound at the active site of myosin V to monitor conformational changes as a function of nucleotide state and the presence of actin. The spin-labeled nucleotides allow us to specifically place a spin probe at the nucleotide site without having to covalently modify the nucleotide site for the more commonly applied approach of site-directed spin labeling. The probe has the spin moiety attached to the 2'-position of the ribose ring (Fig. S2). All X-ray structures of myosin with bound nucleotide show the ribose hydroxyls exposed to solvent and pointing out into the solvent phase. Thus the design of the probes allows them to bind at the nucleotide site without steric interference, while using the more or less restrictive nature of the adjacent nucleotide site to provide structural information. We find that the nucleotide site is closed when myosin V is detached from actin. When myosin V binds to actin in the strongly bound A•MV•ADP state, the nucleotide site opens. The open conformation is in equilibrium with a closed conformation, with the open conformation strongly favored at temperatures approaching physiological. The closed nucleotide site when myosin V is detached from actin and an open site when strongly bound to actin correlate well with the chemomechanical cycle. ATP hydrolysis occurs in the detached state and a closed nucleotide site would be anticipated to better control the required catalytic water. Nucleotide exchange occurs when bound to actin and an open nucleotide site would be anticipated to facilitate this process. Similar observations were previously obtained for rabbit fast and slow skeletal myosin II ^{21,27} and *Dictyostelium* myosin II ²². In this regard, although the physical parameters characterizing the open and closed conformations for myosin II and myosin V are all similar, those for myosin V are closer to those observed in slow skeletal myosin and *Dictyostelium* myosin II. This might be anticipated since both are slower isoforms, and comparison over a wide range of myosin isoforms and speeds of sliding velocities shows the more open form of the nucleotide pocket increasingly favored in slower isoforms ²⁷.

We have previously shown that the equilibrium between open and closed conformations of the nucleotide-binding site in the A•M•SLADP complex are linearly correlated with the sliding velocity generated by the myosin isoform ²⁷. Faster myosins, such as that from insect

flight muscle, favor the more closed conformation, while slower myosins favor the more open conformation. Myosin V is one of the slowest myosins examined, and favors the open state by 4 kJ mol^{-1} at 25°C . The open state is much more favorable than in fast skeletal myosin (1 kJ mol^{-1}) at 25°C , or insect flight muscle, where it is unfavorable by 3 kJ mol^{-1} at 25°C .

Every experimental approach has strengths and weaknesses. X-ray crystallography provides high-resolution structures, but each structure is only a single snapshot of a multi-conformation process, so crucial states may still be missing from the X-ray database. In particular, X-ray structures of the actin-bound states have not been possible. These are the critical states that produce force and motion. The structure of the actin-bound states must instead be inferred from X-ray structures solved in the absence of actin. EPR spectroscopy provides lower resolution structural information, but the data can be accumulated under conditions simulating physiological conditions in the presence and absence of actin. Here we have augmented experimental analysis with computational modeling to bridge the gap between spectroscopy and the X-ray database in order to provide a more complete understanding of the mechanism by which myosin, nucleotide and actin interact to produce force.

Relationship to previously published work

Our quantitative analysis of the experimentally determined spectra relied on the standard cone-angle analysis to approximate the relative open and closed nature of the nucleotide pocket. Although this is a useful approximation, Fig. 3 clearly shows irregularly shaped nucleotide pockets. If X-ray structures are available, an order parameter analysis can overcome the limitations of the cone angle approximation and provide a quantitative measure of the differences in the conformations based on high-resolution data. Importantly however, one can also then correlate an order parameter with the corresponding cone angle that would be experimentally observed for the given x-ray structure (Methods). For the MD simulation of the EPR probe in the closed nucleotide-site myosin V structure, the order parameter, $S_m = 0.62$, determined via computational simulation using the nucleotide-analog spin probe docked in the X-ray structure, would correspond to an experimentally observed spectrum with low-field to high-field splitting equating to a cone angle of mobility of 87° . For the open nucleotide-site simulation with $S_m = 0.22$, the corresponding cone angle of mobility would be 141° . The 87° value is in excellent agreement with the 82° cone-angle value experimentally observed for MV•2'-SLADP (Table 1).

As previously noted, assigning myosin X-ray structures that were solved in the absence of actin as actin-bound states requires an extrapolation that is generally based upon such considerations as the positions of Switch 1 and Switch 2, the orientation of the converter domain, the torsion of the core β -sheet of the protein, and whether the 50 kDa cleft is open or closed. Based on these considerations, Coureux and co-workers²⁴ suggested that the MV•ADP•BeFx structure is the post-rigor, MV•ATP state that results from ATP-binding and cross-bridge detachment at the end of the powerstroke. As they also noted, the conformation of Switch 1 and the remainder of the nucleotide pocket that would interact with the spin-probe moiety in the attached post-rigor state are also very similar to that seen in the X-ray structure previously assigned to be the post-rigor M•ADP•BeF₃ (PDB ID 1MMD, ref.¹⁰) state from the *Dictyostelium* myosin motor domain. The same conclusion holds for the pre-powerstroke M•ADP•VO₄/AlF₄ (PDB ID 1VOM, ref.¹⁴; PDB ID 1MND, ref.¹⁰) conformations of *Dictyostelium* myosin motor domain when detached from actin, as well as in other X-ray structures of chicken fast skeletal myosin³⁰, *Dictyostelium* myosin^{11,31,32}, scallop myosin¹³ and smooth muscle myosin³³. EPR spectra have previously been published from *Dictyostelium* myosin²² with 2'-SLADP docked at the active site. As a further test of the computational approach employed here, we have also

done MD simulations of 2'-SLADP docked at the active site of the X-ray structure of the *Dictyostelium* M•ADP•BeF₃ complex. The simulated order parameter from this simulation, $S_m = 0.69$, corresponds to an experimental EPR spectrum with a low-field to high-field splitting of 5.98 mT or equivalently a cone angle of 77° (data not shown). The value from the MD simulation is again in excellent agreement with experimentally observed cone angles for the spectra of the *Dictyostelium* M•2'-SLADP and M•2'-SLADP•BeFx states, which were 82° and 72°, respectively. Although spectroscopic observation and MD simulations of nucleotide-analog probe mobility have not been done for all the myosin II X-ray structures, the similarities of the nucleotide sites of the X-ray structures, the experimental spectroscopic observations of comparable EPR probe mobilities in the cases examined, and the computational modeling all point to these structures having comparable conformations of the nucleotide pocket. EPR spectroscopy also demonstrates both open and closed conformations of the nucleotide pocket that are in equilibrium for myosin V and myosin II bound to actin. Furthermore as is evident from Figs. 1 and 2, the low-field to high-field splittings for the detached MV•2'-SLADP and MV•2'-SLADP•BeFx/AlFx states are approximately the same as for the more immobilized component in the spectra from the A•MV•2'-SLADP state. A similar observation was made for *Dictyostelium* myosin II²². Probe mobility and computational modeling argue for an actin-bound ADP state with a nucleotide pocket having a conformation similar to that in the detached post-powerstroke state. The existence of this additional state with a closed nucleotide site again points out the difficulty of accurately assigning X-ray structures determined in the absence of actin to actual actin-bound states.

Similar computational approaches have been successfully applied previously. LaConte et al²⁹ used the approach to compare the mobility observed for spin probes covalently attached to the regulatory light chain of muscle fibers with that expected from the X-ray structure of the light chain. They found excellent agreement between experimentally observed mobility and that predicted from the MD simulations. We extended the approach to nucleotide-analog spin probes bound at the nucleotide site of kinesin-family motors²⁸. Open and closed conformations of the nucleotide site are also found in the X-ray database for these motors. These studies found excellent agreement between the experimentally observed probe mobility and the mobility predicted by molecular dynamics modeling of probes docked in the X-ray structures. In the current study we used the closed *Dictyostelium* myosin II as an additional control simulation and found very good agreement between experiment and simulation. Molecular dynamics simulations of the regulatory light chain of smooth muscle myosin have likewise shown excellent agreement with experimental observations³⁴. The molecular dynamics simulation approach has also been extended to reliable modeling of the motion of much larger fluorescent probes³⁵.

The open nucleotide-site X-ray structure has previously been suggested to be a strongly bound A•MV•ADP state at the end of the powerstroke^{24,36}. This assignment is based on the weakened binding of ADP to the A•MV•ADP state relative to the MV•ADP state as would be expected from the extremely wide-open nucleotide pocket seen in Fig. 3, the closed 50 kDa cleft, and the non-planar orientation of the core β -sheet in the X-ray structure. In the EPR spectroscopy experiments, the myosin and actin are combined in solution. Thus there is no external load on the myosin V and the A•MV•SLADP state will assume the minimum free energy state associated with the end of the powerstroke. We find that the probe mobility to be expected from the open MV•ADP X-ray structure state is 35°-40° (~40%) greater than the experimentally observed mobility of the most mobile component seen in the spectrum of the A•MV•SLADP state (141° vs. 99°-106°, Table 1). The X-ray structure was solved in the absence of actin. There is no Mg⁺⁺ at the nucleotide site. However, considering this alternative possibility, the experimentally observed mobilities of the MV•2'-SLADP state and the MV•2'-SLADP, EDTA states are even more restricted. Thus the experimental data

and computational modeling do not support the hypothesis that this is the structure of the primary force-generating A•MV•ADP state at the end of the powerstroke. We cannot rule out the possibility that there is a more mobile actin-bound component to the spectrum. However, if it is, this conformation of the nucleotide-binding site is from a very sparsely populated state representing less than 5% of the actin-bound cross-bridges in A•MV•ADP states. This is the limit of our resolution in resolving distinct spectral components²⁷. As the nucleotide-binding sites of all myosin X-ray structures fall either into the closed-Switch 1 conformation seen in the post-rigor state, or in the open-Switch 1 conformation seen in the myosin V structure, EPR spectroscopy suggests that the structure of the force-producing A•MV•ADP state at its free energy minimum has yet to be resolved by X-ray diffraction. We emphasize that these results do not preclude the open X-ray structure from representing an actin-bound state. Myosin V is dimeric and processive. Head-head mechanical interaction result from two simultaneously bound heads has been proposed to modulate myosin V kinetics³⁷⁻³⁹. The myosin V construct used in these studies is monomeric and thus would miss an open state that has an obligatory mechanical component.

In summary, the conformational changes at the nucleotide site of myosin V are similar to those previously observed for other myosin isoforms, with a closed nucleotide pocket in myosin alone. There is an equilibrium between an open and closed conformation in the A•MV•SLADP state, with the open conformation becoming more populated with decreasing temperature. MD simulation of EPR probe mobility provides a mechanism to bridge the gap between spectroscopy and X-ray crystallography. Experimentally observed probe mobility in MV•SLADP state without bound actin is similar to the simulated probe mobility observed in the closed myosin V X-ray structure. The experimentally observed probe mobility in the A•MV•ADP state and simulated mobility of the X-ray structure of the A•MV•ADP state differ too dramatically for them to represent the same conformation of myosin seen in the open X-ray structure.

METHODS

Protein Cloning and Purification

The first 770 amino acids of Chicken MyoVa were cloned into pFastBac. For purification the construct has an N-term 6X His tag, followed by TEV protease recognition site. Bacmid generated in Sf-9 cells followed by three rounds of virus amplification. For protein expression, 2L of Sf-9 cells at 2×10^6 cells/ml (>99% viability) were infected with 7% virus (moi 1). The culture was grown at 28° C with gentle shaking for 60 hours, until the cell viability decreased to 78%. Cells were pelleted by centrifugation ($4500 \times g$) and re-suspended in a lysis buffer (50mM Sodium Phosphate, pH 8, 10mM Imidazole, 300mM NaCl, 4mM MgCl₂, 1mM DTT and protease inhibitors (1ng/ml aprotinin, pepstatin, leupeptin; 50uM PMSF). The cell lysate was centrifuged at $30500 \times g$ for 40 minutes. The supernatant was applied to Ni-NTA resin (Qiagen). The column was washed with 5 column volumes of 50mM Sodium Phosphate, pH 8, 20mM Imidazole, 600mM NaCl, 4mM MgCl₂. The protein was eluted with 50mM Sodium Phosphate, pH 8, 250mM imidazole, 300mM NaCl, 4mM MgCl₂. The protein was purified off the Ni-NTA resin, fractions were pooled and applied to a HiTrap Q column Sepharose HP 5-ml column (Amersham). 0% Salt Buffer: 10mM Tris, pH 8, 4mM MgCl₂, 1mM DTT; 100% Salt Buffer is the same + 1M NaCl. Gradient went from 0% salt to 100% salt over 20 column volumes. Fractions from the Q column were pooled; the protein was applied to a gel filtration column (HiLoad 16/60 Superdex 200, buffer used was 10mM Tris, pH 8, 300mM NaCl, 4mM MgCl₂, 1mM DTT). Fractions from this column were pooled, concentrated and then dialyzed into Assay Buffer (25 mM KCl, 25 mM imidazole HCl, pH 7.4, 1 mM EGTA, 4 mM MgCl₂ and 10 mM DTT) with 50% (w/v) glycerol.

EPR spectroscopy

Myosin V motor domain was exchanged into the rigor buffer (5 mM Mg(OAc)₂, 1 mM EGTA, 40 mM MOPS, pH 7.0) concentrated to 100 μ M using a centricon concentrator, and then labeled by the addition of 75 μ M 2'-SLATP. The triphosphate species is hydrolyzed by myosin to the diphosphate species ²¹. The labeled protein was then run through a Micro Bio-Spin column P-30 (Bio-Rad) spin-column to eliminate free probe. EPR spectra were obtained in a 50- μ l glass capillary. For experiments with actin, the labeled myosin was added to F-actin at a stoichiometry of 1:10 myosin heads/actin monomers in the rigor buffer. This mixture was centrifuged at 70,000 $\times$ g for 20 min. The pellet containing the actomyosin complex was transferred to a quartz flat cell. The actin concentration in these pellets was estimated at 600 μ M. The pellet was covered with a glass coverslip, sealed with vacuum grease to prevent dehydration, and placed in the EPR cavity. The spin-labeled nucleotide 2'-SLATP (Fig. S2) was synthesized using slight modifications of previously published methods ⁴⁰.

EPR measurements were performed with a Bruker EMX EPR spectrometer (Billerica, MA). First derivative, X-band spectra were recorded in a high-sensitivity microwave cavity using 50s, 10 mT-wide magnetic-field sweeps. The instrument settings were as follows: microwave power, 25 mW; time constant, 164 ms; frequency, 9.83 GHz; modulation, 0.1 mT at a frequency of 100 kHz. Each spectrum used in data analysis was an average of 10–50 sweeps from an individual experimental preparation. Temperature was controlled by blowing dry air (warm or cool) into the cavity and monitored using a thermistor placed close to the experimental sample.

Effective cone angles of mobility can be approximated using the order parameter $S = (T_{\parallel}' - T_0)/(T_{\parallel} - T_0)$ as a measure of probe mobility. Here $2T_{\parallel}'$ is the observed splitting, $2T_{\parallel}$ is the splitting for an immobilized probe, and $2T_0$ is the isotropic hyperfine splitting for freely tumbling SLADP in solution. Then the cone angle is then given by $\cos \theta = -0.5 \pm 0.5 \cdot (1 + 8S)^{1/2}$, where 2θ is the vertex angle of the cone of mobility. Additional details are in ^{25,41}.

Molecular dynamics simulations

The atomic-level protein structures of MV•ADP•BeF₃ with a closed phosphate tube (PDB ID 1W7J, ref ²⁴) and MV•ADP with an open phosphate tube (PDB ID 1W7I, ref ¹⁸) were the starting points for the MD simulations. The neck was truncated at amino acid 764. The light chain and the BeF₃ moiety were eliminated by editing the Protein Data Bank file. 2'-SLADP was docked at the nucleotide site of the remaining, truncated MV•ADP structures via a superposition of the ADP moiety in the X-ray structure and the ADP moiety of 2'-SLADP. This put the ADP moieties in an identical location and resulted in the ribose hydroxyls of 2'-SLADP pointing out of the nucleotide site into the solvent phase. The spin moiety of 2'-SLADP is at the 2'-position of the ADP ribose. Thus the docking also results in the spin moiety pointing out into the solvent phase. Additional details are in ⁴². Simulations were done using the Amber10 suite of codes ⁴³. Hydrogen atoms were added to the structure using the tleap module of Amber. Charges for 2'-SLATP were derived by first performing a single-point energy calculation using Gaussian03 ⁴⁴ to obtain electrostatic charges. These are then fit to the molecules by using the RESP procedure ⁴⁵. Van der Waals parameters for the Mg²⁺ that cofactors the nucleotide in the closed MV•ADP structure are taken from ⁴⁶ and adjusted as described in ⁴². The open structure has no Mg²⁺ ion co-factored to the nucleotide. Equilibrium bond lengths and angles for the spin-ring moiety were averages of the values in ^{47,48}. The nitroxide bond was modeled as having sp² hybridization. The generalized Born approach was used for the MD simulation (reviewed in ⁴⁹). The structures were first energy minimized using 1000 steps of the steepest-descent method, followed by

500 steps of conjugate-gradient minimization. The RMS deviation of the structures over the last 500 steps was less than 0.02Å. The MD simulation was done at 298K with temperature was maintained via coupling to an external bath using the Berendsen algorithm⁵⁰. The SHAKE algorithm⁵¹ was employed for numerical integration in time. The MD-simulated motions of the protein and spin-probe moiety were followed for 14 ns using a 2 fs time step for the simulations of myosin V with a closed nucleotide pocket. Naber et al²⁸ have shown this is sufficient simulation time for a spin probe to sample the available space via thermal motion in a restricted space. For the open conformation, it was necessary to simulate for 75 ns for the probe to have sufficient time to sample the larger spatial region. The aggregate angular distribution of the probe, $\rho(\theta)$, did not change for either simulation over the last 5ps of the simulations, implying convergence. The EPR spectrum is dependent on the angular deviation of the p-orbital of the nitroxide bond from a fixed direction in the laboratory reference frame (Figure S3). The p-orbital is perpendicular to the plane of the C-N-C bond of spin moiety ring at the nitroxide nitrogen. During the MD simulation, the probe executes a pseudo-random walk as a function of time (Figure S4)²⁵. The directional vector perpendicular to the plane of the C-N-C bond of spin moiety ring was output every 4 fs to a trajectory file for analysis of the simulated mobility of the probe.

The angular distribution of the directional vector was binned in 1° increments to approximate the probability density, $\rho(\theta)$, as a function of angular deviation, θ , from the mode vector of the angular distribution. Assuming rapid motion of the probe, the order parameter for the modeled mobility from the simulation is then given by and the spin label order parameter is given by

$$\langle \cos^2 \beta \rangle = \frac{\int_0^\pi \rho(\theta) \cos^2(\theta) \sin(\theta) d\theta}{\int_0^\pi \rho(\theta) \sin(\theta) d\theta} \quad (1)$$

and the spin label order parameter is given by

$$S_m = 1.5 \langle \cos^2 \beta \rangle - 0.5. \quad (2)$$

Thus a spin probe undergoing rapid, uniformly distributed motion within a cone with full vertex angle $C_\angle = 2 \arccos[-0.5 + 0.5(1 + 8S_m)^{1/2}]$ would have the same order parameter, S_e , based on the experimentally observed spectrum. This allows comparison between cone angles of mobility determined experimentally from EPR spectra and the order parameter determined from the MD simulations. Additional details are in^{25,28}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grants HL032145 (T.P.), AR042895 (R.C., N.N.), GM33289 (S.S.) and GM077067 (E.P., N.N.). We thank James Spudich and Alex Dunn for generously providing the myosin V used in this work.

Abbreviations used

MV myosin V

A	actin
MD	molecular dynamics
SLADP	2'-spin labeled ADP
EPR	electron paramagnetic resonance

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Figure 1. EPR spectra of MV•2'-SLADP (blue), MV•2'-SLADP in the presence of excess EDTA over Mg^{++} (cyan), MV•2'-SLADP •BeFx (red), and MV•2'-SLADP•AlFx (black). All spectra were accumulated at 23°C.

Figure 2. EPR spectra of A•MV•ADP at (A) 34°C (cyan), (B) 23°C (red), (C) 12°C (black), (D) 2°C (green) and (E) MV•ADP, 23°C (blue).

Figure 3.

Overlay of ribbon diagrams of the X-ray structures of myosin V with a closed (blue) and open (coral) nucleotide-binding site with 2'-SLADP docked at the site. ADP is in red, except for the spin ring moiety, which is dark gray, and the nitroxide, which is in cyan. The P-loop (gray), Switch 1 (magenta) and Switch 2 (green) are shown. Switch 1 and a surface loop (amino acids 308-310, yellow) restrict probe mobility in the closed conformation. They are displaced away from the spin moiety by 8-10 Å toward the right side of the figure in the open conformation allowing for greater probe mobility.

Figure 4.

Simulated trajectory of 2'-SLADP at the nucleotide site of myosin V with a closed nucleotide pocket. The cyan background is a CPK rendering of the X-ray structure of myosin V with a closed nucleotide pocket. The nucleotide is at center with standard atom colorings, except for the nitroxide, N-O, bond, which is in purple. The adenine ring is at lower left. The spin moiety explores the available space as a function of time. An order parameter for the simulation can be calculated from the trajectory and compared with experimental observation. The probe orientations shown were selected to emphasize the variability of probe orientation. They are not a representative distribution (see Fig. 5).

A

B

Figure 5.

Angular orientation of the nitroxide spin probe in MD simulations of 2'-SLADP bound at the active site of the X-ray structure of myosin V with (A) an open and (B) a closed nucleotide site. The points on the plot are equally spaced in time over a 14ns simulation time. For clarity of presentation, there are 6999 MD-simulated time points between each time point shown on the plots that are omitted from the plots.

Figure 6. Orientational probability distribution, $\rho(\theta)$, for the simulation of 2'-SLADP bound at the active site of the X-ray structure of myosin V with an open (red) and a closed (blue) nucleotide binding site. The order parameters and effective cone angles for the distributions are given.

Table 1

Summary of low-field to high-field splittings and equivalent cone angles for 2'-SLADP bound to myosin V and actomyosin V. The splitting for the A•MV states is for the major component visible in the spectrum. Splittings are the mean of 3-6 different observations. Standard errors of the EPR splittings were ± 0.02 mT. The last column gives the conformation of the nucleotide site.

State	Splitting (mT)	Cone Angle (°)	Binding Site
MV•ADP 23°C	5.9	81.7	Closed
MV•ADP, EDTA 23°C	5.9	81.7	Closed
MV•ADP•BeFx 23°C	6.1	72.2	Closed
MV•ADP•AlFx 23°C	6.1	72.2	Closed
A•MV•ADP 2°C	5.4	99.0	Open
A•MV•ADP 12°C	5.3	102.3	Open
A•MV•ADP 23°C	5.2	105.0	Open
A•MV•ADP 34°C	5.1	106.2	Open