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Conformational changes at the nucleotide pocket of motor proteins monitored by electron paramagnetic resonance spectroscopy*

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Abstract: A fundamental goal in the field of motor proteins is to identify the conformational changes associated with the hydrolysis of the physiological substrate, ATP, and to define how these conformational changes are modulated by binding to the polymer track and translated into biologically useful movement. We have used electron paramagnetic resonance (EPR) spectroscopy to monitor conformational changes at the nucleotide-binding site of myosin- and kinesin-family motors. A novel set of nucleotide-analog EPR spin probes were synthesized and used to localize a spin moiety at the nucleotide site. This allows a reporter group to be placed with high specificity at the ATP binding site. Our results indicate that the nucleotide-binding site of myosin motors opens when the motor binds to its polymer roadway, actin, while that of kinesin closes on binding to microtubules (MTs). However, the transition is not all-or-none. There is instead an equilibrium between open and closed conformations. The different conformational changes in the two motor families can be correlated with differences in their biochemical cycles. Thus, we can now define the relationship between nucleotide-site structure, biochemistry and polymer binding for the two motors.

Keywords: actin; electron paramagnetic resonance spectroscopy; kinesin; myosin; nonclaret disjunctional (ncd) protein; nucleotide spin probes; switch 1.

INTRODUCTION TO THE MOTOR PROTEINS

Motor proteins are a class of molecular motors that are responsible for all forms of movement in the cellular world. In the cytoskeleton, the two most prominent examples are the muscle protein myosin II, which powers the contraction of muscle fibers in animals, and the kinesins, which play essential roles in intracellular transport such as axonal transport and in the formation of the spindle apparatus and the separation of the chromosomes during mitosis and meiosis. Motor proteins use chemical energy resulting from the hydrolysis of ATP to move unidirectionally along a polymer track. Myosin translocates along actin filaments, kinesin along microtubules (MTs). The direction of the movement depends on the structural polarity of the track. In the kinesin-family motors, some motors move toward the positive end of the MT such as kinesin 1 and other proteins such as nonclaret disjunctional (ncd) move toward the

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negative end of the MT. Both motor families are active areas of research for therapeutic agents that could enhance or inhibit activity as a prophylactic treatment for disease states.

Kinesin and myosin have dramatically different sizes (myosin 850 aa, kinesin 350 aa, respectively), no significant amino-acid identity, and have different motile properties. Thus, it was truly a surprise when the initial X-ray structures of myosin [1] and kinesin [2,3] were compared. Alignment of the two proteins showed a strong structural overlap between kinesin and the core domain of myosin. Indeed, the overlap also extends to the G-proteins [4,5], suggesting evolution of all three from a common ancestor protein [6]. In particular, the phosphate-binding domain of all three was composed of the P-loop, switch 1, and switch 2 motifs initially described in the G-proteins. However, it was also noted early on that if one took a closer look at the ATP binding site of the initial X-ray structures, there was a significant difference in the switch 1 domain [7]. In myosin, switch 1 was located directly adjacent to the nucleotide triphosphates, forming with the P-loop and switch 2 a closed "phosphate-tube" structure tightly enclosing the triphosphates [8]. In kinesin, switch 1 was in a very different position, pulled away from the triphosphates, leaving them exposed to solvent (Fig. 1). Structural and functional similarities frequently suggest common enzymatic mechanisms. The difference led us to ask if myosin and kinesin are showing different open and closed nucleotide-site conformations that exist in both proteins. X-ray crystal structures are static snapshots in time of a single structure, and key snapshots may be missing in the database. Additionally, X-ray structures of the motors bound to their polymer roadways continue to elude investigators. Although providing lower-resolution structural data, EPR spectroscopy has provided valuable information on the dynamics of the structures of the motor proteins both alone and when attached to their polymers, which is unattainable by crystallography.

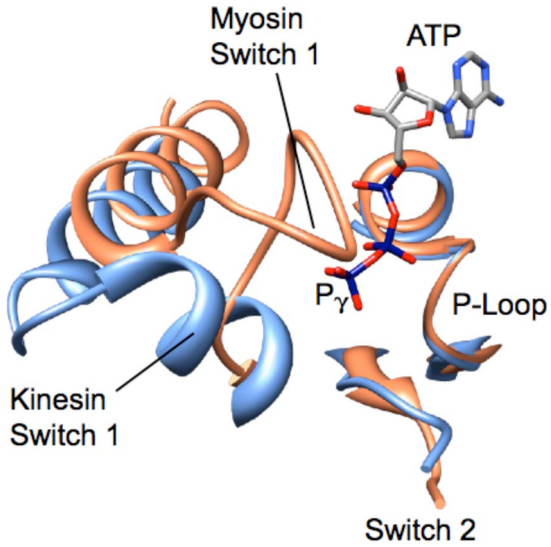

Fig. 1 Overlay of the nucleotide-binding domains of the X-ray structures of myosin (Dictyostelium myosin·ADP·BeF_3, PDB ID 1MMD, ref. [11]) and kinesin (kinesin·ADP, PDB ID 1BG2, ref. [2]). The P-loop and switch 2 superimpose. Switch 1 in myosin (coral) is a loop adjacent to the nucleotide phosphates. In kinesin (blue), switch 1 is displaced approximately 8 Å away from the nucleotide, creating a trough-like binding pocket in which the nucleotide rests. The ATP is ADP·BeF_3 from the myosin·ADP·BeF_3 structure [11] with the BeF_3 moiety changed to the γ-phosphate moiety.

EPR SPECTROSCOPY AS A TOOL TO STUDY MOTOR PROTEINS

Many biophysical and biochemical techniques have been used to study the conformational changes in macromolecules, including NMR, electron microscopy, and fluorescence spectroscopy. We used EPR spectroscopy to investigate the conformational changes at the ATP binding site of myosin and kinesin. EPR has the advantages that the experimental approach can be used under conditions mimicking physiological in both the presence and absence of polymer. Multiple conformations can also be simultaneously monitored, and the thermodynamics of the transition between the different protein conformations can be analyzed.

EPR detects species with unpaired electrons, such as free radicals, many transition metals, and reaction intermediates. It is similar to NMR except that in EPR it is the electron spins that are excited instead of the spins of the atomic nuclei. The majority of proteins do not contain free radicals, and therefore they are created artificially. Nitroxide spin labels are specially designed nonreactive radical molecules that have been widely used in EPR spectroscopy of biological molecules, Fig. 2. These are nitroxide derivatives with a stable unpaired electron in the p-orbital of the N–O bond. The nitroxide radical is stabilized by the presence of two methyl groups on each of the adjacent carbon atoms. To limit their flexibility, the nitroxide bond is usually enclosed into a 5- or 6-membered ring structure. Nitroxide spin labels are attached to the protein covalently through a side chain of an amino acid, usually on a cysteine residue. They can also be attached to the protein noncovalently as analogs of enzyme substrates or cofactors.

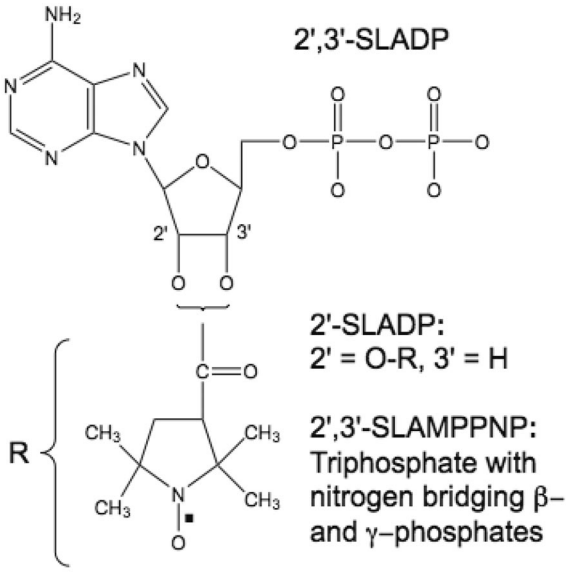

Fig. 2 Structures of nucleotide-analog EPR probes used in these studies. The nitroxide spin probe is shown attached to the 2'- and 3'-hydroxyls of the ribose ring. The unpaired electron is shown as a dot adjacent to the nitrogen on which it is localized.

When placed in a strong magnetic field, the unpaired electrons give rise to a spectrum. The EPR spectrum of a probe attached to a protein is sensitive to the local environment of the probe. The mobility of the probe is determined by the adjacent surface of the protein. If the probe is moving freely in solution, the resulting EPR spectra consists of three sharp lines that are produced by the interaction of the electron spin with the nuclear spin of the nitrogen atom on which it is localized, see Fig. 3. When the probe is attached to a protein, the surface of the protein restricts the mobility of the probe, which

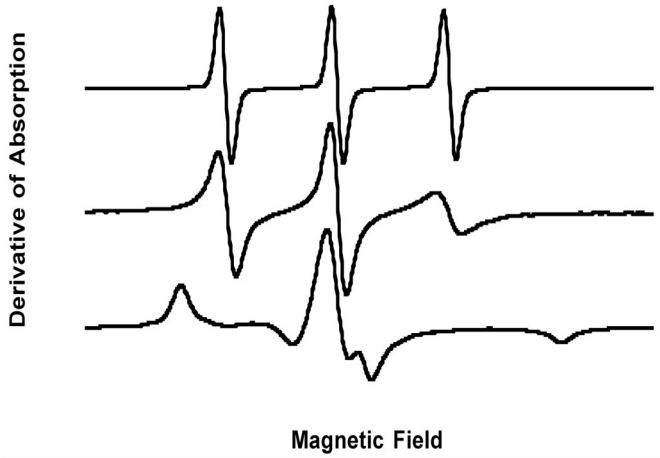

Fig. 3 Examples of EPR spectra for different degrees of immobilization. The derivative of absorption is shown as a function of the magnetic field. The derivative of absorption is observed rather than absorption for technical reasons. The width of the field is 9.3 mTesla. The top spectrum is for a free probe tumbling with sub-nanosecond correlation times. The middle spectrum is for a probe weakly immobilized by the protein surface undergoing rapid rotations within a cone with vertex angle of 70º. The bottom spectrum is for a probe that is completely immobilized by the protein, with a rotational correlation time of greater than a millisecond.

will result in a broadening of the spectral peaks and the outward movement of the low- and high-field peaks. The more restricted the region of motion is, the greater is the low- to high-field splitting to the point of a motionless probe. Griffith and Jost [9] showed that the mobility of the probe on the surface of the protein can be modeled as a motion in a cone with a vertex angle that can correlated with the magnitude of the splitting between the low-field peak and the high-field dip. The protein surface is most certainly not a cone of revolution, but the analysis serves as a first-order approximation to correlate changes in the magnetic field available with change of the restrictive nature of the protein surface.

In this work, we are interested in the conformational changes at the nucleotide-binding site of the motor proteins kinesin and myosin. The traditional approach of site-directed spin labeling requires producing proteins with only a single cysteine at the location of interest, which is then covalently modified by reaction with the spin probe [10]. This is a powerful method since it allows cysteine scanning where each residue along a polypeptide chain can be changed to a cysteine and labeled with a nitroxide spin probe. However, covalent modification can perturb protein function. Here we instead use a novel set of ATP-analog spin probes to specifically place a probe at the ATP binding site, shown in Fig. 2. We attached the spin probe to the ribose moiety of ATP at the 2'- or the 3'-hydroxyl groups (2',3'-SLATP). Alternatively, the attachment is through the 2'- or the 3'-position of the ribose ring, while the other position is occupied by a hydrogen replacing the hydroxyl group to generate the deoxy spin probe derivatives (2'-SLATP and 3'-SLATP). A spin probe was also attached to the ribose hydroxyls of the nonhydrolyzable ATP analog AMPPNP to generate 2',3'-SLAMPPNP. AlFx and BeFx were used to mimic the ADP·Phosphate states [11].

MONITORING THE CONFORMATION OF THE NUCLEOTIDE SITE USING SPIN-LABELED NUCLEOTIDES

Motor proteins were incubated with the spin-labeled nucleotides, and excess unbound probes were removed by chromatography. Figure 4 shows EPR spectra demonstrating the basic results obtained in these experiments. Figure 5 shows the steps in the ATPase cycle that will be selectively populated using different conditions for the EPR experiments. Except as noted, all spectra were accumulated at 23 °C.

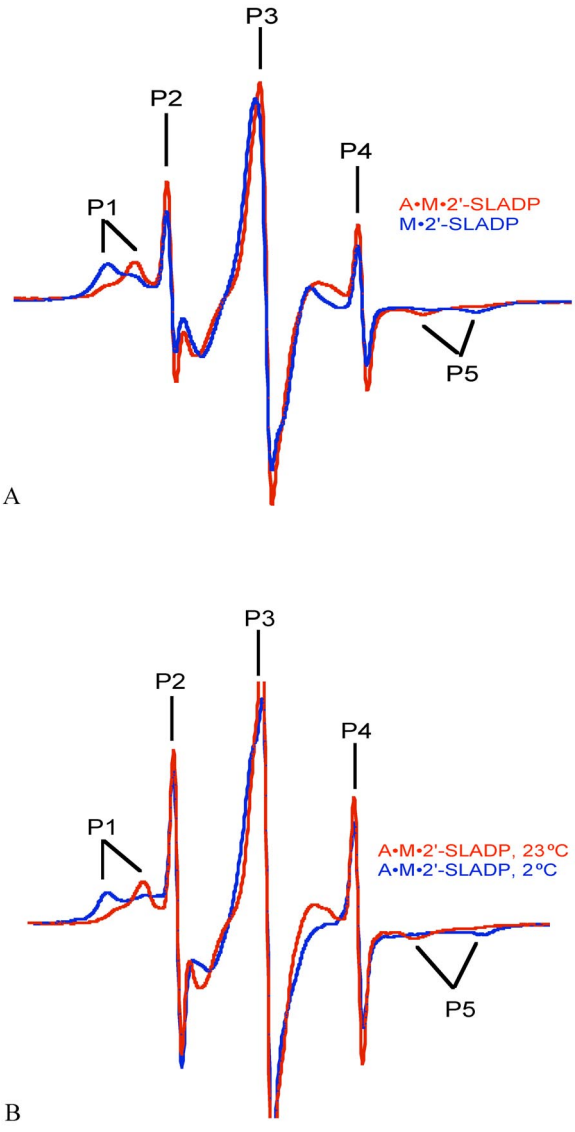

Fig. 4 EPR spectra of 2'-SLADP bound to the ATP binding site of rabbit skeletal myosin. The horizontal axis is magnetic field. The vertical axis is the derivative of absorbance. (A) M·2'-SLADP (red), A·M·2'-SLADP (blue). (B) A·M·2'-SLADP, 23 °C (red), A·M·2'-SLADP, 2 °C (blue). Spectra are 10.0-mTesla-wide sweeps with center-field at 350.1 mTesla.

Figure 4A (blue) is the spectrum of rabbit skeletal myosin motor domain bound to 2'-SLADP (M·2'-SLADP). The spectra show a mixture of states with different probe mobilities. The three sharp central peaks, P2, P3, and P4, arise primarily from unbound spin probe tumbling rapidly in solution. When the probe binds at the active site of myosin, the adjacent protein surface determines the region in space that the probe can explore in the presence of thermal fluctuations. The restriction on the available space for motion now results in a broadening of the spectrum. The resulting low-field peaks, P1, and high-field dips, P5, are from spin probe bound at the active site of myosin. The more restricted the region of motion is, the greater is the low- to high-field splitting. The low- to high-field splitting is 6.4 mTesla. As previously noted, EPR probe mobility can be modeled as motion in a cone of revolution

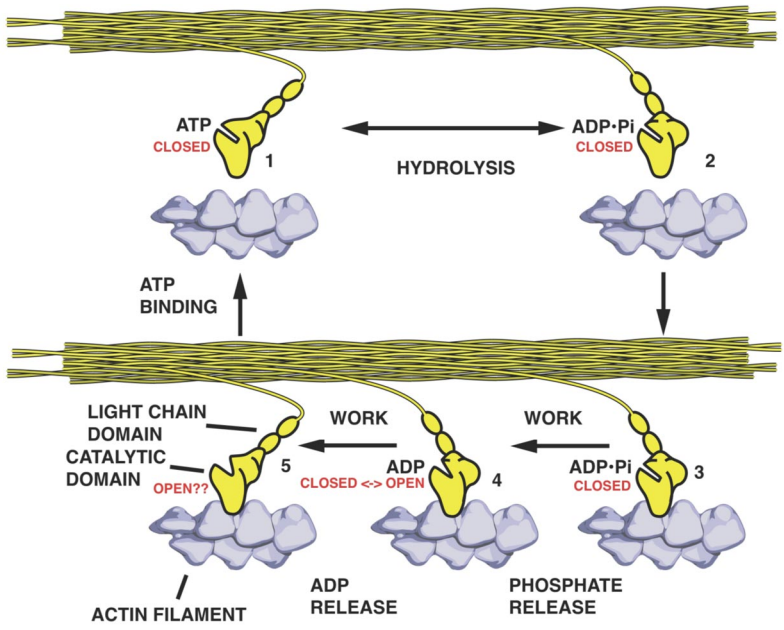

Fig. 5 Cartoon linking the actomyosin ATPase cycle to the mechanics of the actomysoin interaction and the conformation of the nucleotide site. Myosin cross-bridges (yellow) project from the thick filament. They can be detached from actin (top row, states 1 and 2) or attached to actin and producing force (bottom row, states 4 and 5). Initial attachment to actin is in a weakly bound intermediate state (state 3) that produces little force. One ATP is hydrolyzed per cycle.

[9]. The change in splitting of M·2'-SLADP, Fig. 4A, corresponds to a vertex angle of the cone angle of mobility of 60º. This is restricted motion representing a closed nucleotide site. Similar results were obtained for the spectra of M·2'-SLADP·AlFx and M·2'-SLADP·BeFx. These two states represent triphosphate-analog states. Figure 5 summarizes the relationship between the actomyosin biochemical cycle, force production, and conformational changes at the nucleotide site. The M·2'-SLATP and M·2'-SLADP·AlFx/BeFx states are detached from actin (states 1 and 2, top). We conclude that the actin-detached states of myosin have a closed nucleotide pocket.

The initial attachment of myosin to the actin polymer is in a weakly bound pre-powerstroke state with the triphosphate (ATP or ADP·Pi) still at the nucleotide site. AMPPNP is a nonhydrolyzable ATP analog. The spectrum from the weakly bound A·M·2',3'-SLAMPPNP is similar to the spectra of M·2'-SLADP·AlFx/BeFx. There has been little change in the P1-P5 splitting of the spectrum when compared to the detached states in Fig. 4A, blue. Thus, the weakly bound state (Fig. 5, state 3) also has a closed nucleotide pocket. The release of phosphate from the weakly bound, pre-powerstroke state results in a transition into a strongly bound, force-producing, powerstroke A·M·ADP state. This is the working actomyosin cross-bridge state (Fig. 5, state 4). Figure 4A, red, shows the spectrum at of the A·M·ADP state at 23 °C. It is clear that there are new P1 and P5 components with reduced splitting (5.0 mTesla) corresponding to a cone angle of 110º. Equivalently, the EPR probe reports a significant opening of the nucleotide pocket. However, the spectrum is more complex in that there remains a more immobilized component with splitting roughly equivalent to that seen in Fig. 4A, blue. In other words, the open conformation of the nucleotide pocket is in equilibrium with a more closed conformation.

The equilibrium between closed and open states is temperature-dependent. The temperature dependence of this equilibrium allows one to measure the changes in enthalpy and entropy associated with the transition from open to closed states. A series of spectra of A·M·ADP was taken at different

temperatures. Figure 4B shows the spectra of actomyosin at 23 °C (blue) and at 2 °C (red) with an increased fraction of the more closed component of the spectrum. We note that replacing 2'-SLADP with the other spin-label ATP analogs resulted in similar results, showing that we are observing changes in protein structure, rather than a local interaction between the probe and the protein. Thus, the observation that the nucleotide site is closed when myosin is not bound to actin, and opens when myosin binds to actin, is a general result and is not dependent on the particular spin probe employed.

The kinetic cycle of kinesin-family motors likewise cycles between states that are detached and attached to their polymer roadway, MTs. Figure 6 shows spectra obtained from the kinesin-family motor, ncd. The spectrum of ncd•2',3'-SLADP (red) shows a P1-P5 splitting of 4.8 mTesla, corresponding to a cone angle of mobility of 118º. The spectra of the other detached, triphosphate-analog states, ncd•2',3'-SLADP•BeFx/AlFx, have similar splittings (data not shown). Thus, we conclude that the situation is exactly the opposite of myosin. Detached kinesin-family motors have open nucleotide pockets as is seen in the original X-ray structures of kinesin [2] and ncd [3]. The spectrum of the MT-bound MT•ncd•2',3'-SLADP state (Fig. 6, black) shows two bound components (seen most easily in the presence of two P5 dips). One has splitting roughly equivalent to that observed in the detached states. The other has greater splitting, implying a closing of the nucleotide pocket. In the triphosphate-analog, MT•ncd•2',3'-SLADP•BeFx/AlFx states, there is a further closing of the nucleotide site. We again note that similar results are obtained for conventional kinesin and for both ncd and kinesin using other nucleotide-analog spin probes [12].

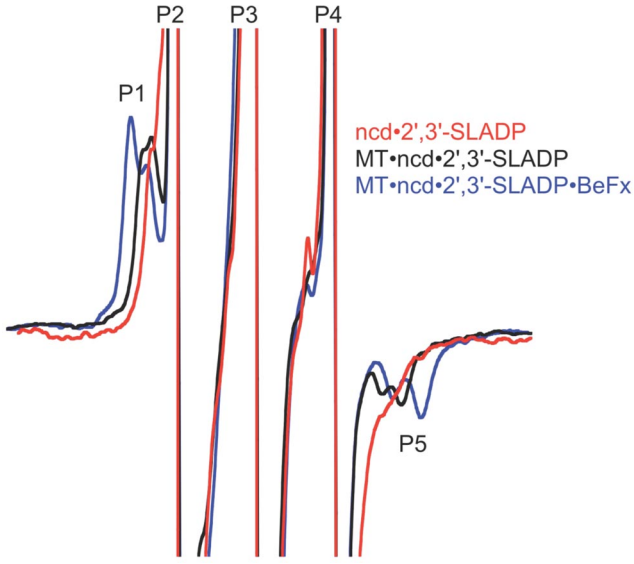

Fig. 6 EPR spectra from the kinesin-family motor, ncd. ncd•2',3'-SLADP (red), MT•ncd•2',3'-SLADP (black), MT•ncd•2',3'-SLADP•BeFx (blue). Spectra are 10.0-mTesla-wide sweeps with center-field at 350.1 mTesla.

Our studies were motivated by the initial observation that despite structural homology, the initial X-ray structures of kinesin-family motors [2,3] and myosin motors [11] showed open and closed nucleotide pockets due to a displacement of switch 1. Figure 7 shows space-filling models of (A) kinesin•ADP (PDB ID 1BG2 [2]) and (B) *Dictyostelium* myosin•ADP•BeFx (PDB ID 1MMD [11]) with 2'-SLADP docked at the nucleotide site. The switch 1 domain is colored coral. Using spin-probe mobility as the reporter signal, we have monitored the opening and closing of the nucleotide pockets of both classes of motors. It is clear that switch 1 will be considerably more restrictive of probe mobility

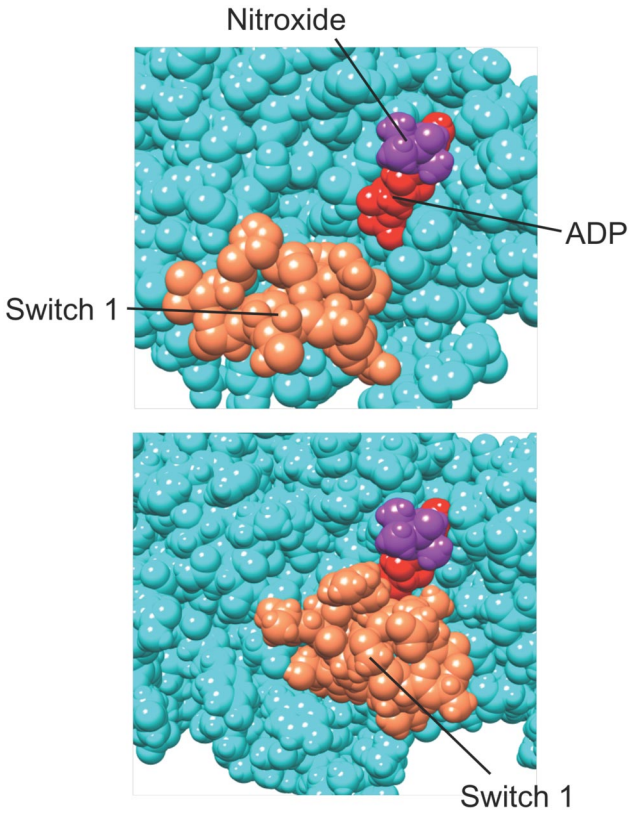

Fig. 7 Space-filling models of the X-ray structures of kinesin·ADP (PDB ID 1BG2 [2] and Dictyostelium myosin·ADP·BeFx (PDB ID 1MMD, [11]) with 2'-SLADP docked at the nucleotide site following [13]. The ADP moiety of 2'-SLADP is colored red. The spin-ring moiety is purple. The protein is colored cyan except for the Switch 1 domain, which is colored coral. In the open conformation of switch 1 (A), the switch is pulled away from the spin moiety and a mobile probe would be anticipated. In the bottom panel (B), the switch is directly adjacent to the spin moiety and would be anticipated to severely restrict mobility.

in the bottom panel where it is directly adjacent to the spin-probe moiety (purple) than in the top panel. Although there are multiple conformational changes at the nucleotide site associated with the opening/closing of the nucleotide site in the X-ray structures, conformational changes in switch 1 would appear to be the only ones that are sufficiently large to generate the EPR probe mobility changes we observe. Additionally, molecular dynamics simulations of EPR probes docked into open and closed nucleotide-site structures demonstrate changes in mobility comparable to those observed experimentally here associated with switch 1 movements [12].

An increasingly large body of evidence from a wide variety of experimental techniques does link switch 1 to the conformational changes we are observing. Molecular dynamics simulations employing homology modeling with myosin initially showed that the kinesin-family motors could have a closed-switch 1 conformation with either a diphosphate or triphosphate substrate at the nucleotide site [13], structures not seen in the X-ray database at that time. An X-ray structure of a virtually identical switch 1 conformation was recently demonstrated with AMPPNP at the nucleotide site of the kinesin-family motor, Eg5 [14]. Sindelar and collaborators have used electron microscopy (EM) reconstructions to show a closed nucleotide site of kinesin when bound to MTs that results from a movement of switch 1 [15]. Additionally, an EPR probe specifically attached to switch 1 of kinesin reports conformational

changes associated with binding to MTs [16]. Likewise for myosin motors, the X-ray structure of *Dictyostelium discoideum* myosin complexed with dynamin [17] and the subsequent structure of myosin V [18] both showed an open nucleotide site that results from a displacement of switch 1. A similar switch conformation was found in EM reconstructions of myosin bound to actin [19]. Fluorescence spectroscopy of an introduced tryptophan in switch 1 of *Dictyostelium* myosin has likewise suggested switch 1 conformational changes associated with an opening of the nucleotide site [20,21]. Thus, a considerable body of evidence directly links switch 1 to the opening and closing of the nucleotide sites of myosin and kinesin that is being reported here by EPR spectroscopy.

The most interesting observation is that the partner filament-induced conformational changes at the nucleotide site of the two motor proteins are opposite each other. Kinesin motors show an open nucleotide site when free from polymer that closes on binding to polymer. Myosin is exactly the opposite. The biochemical cycle of myosin is unusual in that ATP hydrolysis occurs when myosin is detached from actin (the biochemical cycle of myosin is reviewed in [22,23]). In Fig. 5, this is the transition from the M·ATP state to the M·ADP·Pi state (state 1 → state 2). Hydrolysis is thought to occur via an inline water attack on the γ-phosphate moiety. This requires precise spatial control of the attacking water. This cannot be accomplished with an open nucleotide pocket, instead requiring a closed phosphate tube [13,14] indicative of the closed nucleotide-binding site reported by EPR spectroscopy. Conversely, ADP release and nucleotide exchange occurs when myosin is bound to actin. Thus, the nucleotide pocket would be expected to open when myosin binds strongly to actin, decreasing the affinity of the actomyosin complex for hydrolysis products and facilitating ADP release (Fig. 5, state 3 → state 4). We do not know the conformation of the nucleotide site in the rigor A·M state (Fig. 5, state 5) at the end of the powerstroke. However, the requirement for ATP-binding to release the cross-bridge and subsequent ATP hydrolysis (Fig. 5, state 5 → state 1 → state 2) would suggest an open conformation for the A·M state.

In the biochemical cycle of kinesin-family motors, the situation is completely different. ATP-binding is instead associated with rapid ATP hydrolysis in the MT-bound complex (reviewed in [24]). This would be facilitated by a closed nucleotide site. Thus, the structural and biochemical observations of hydrolysis steps that are 180º out of phase are consistent with myosin hydrolysis when free from actin in a closed phosphate-tube structure and kinesin hydrolysis in a closed phosphate-tube structure when bound to MTs.

CONCLUSIONS

Spin-labeled nucleotides have provided a powerful method for monitoring the conformation of the nucleotide-binding sites of myosin and kinesin motors. The spectra can be easily deconvolved into three components, free nucleotide and nucleotides bound to the motors in two different conformations. The data have shown that these sites change their conformation when the motors bind to their polymer roadways. A more quantitative interpretation of EPR spectra can be made by modeling the mobility expected for spin probes docked to known high-resolution structures of the motors, using molecular dynamics [12,13,25]. Correlations between EPR spectra and crystal structures using molecular dynamics suggests that the conformational changes detected by the EPR spectra involve movements of the switch 1 region of the motors. This analysis has allowed us to interpret the spectral changes with the cycle in terms of the molecular mechanism of motility for both motors. The data suggest that the nucleotide site of myosin motors opens upon binding of myosin to actin to facilitate the release of nucleotides. In contrast, the nucleotide site of kinesin motors closes upon binding to MTs to facilitate the hydrolysis of nucleotides.

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