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ANALYSIS OF THE INTERACTION OF THE EG5 LOOP5 WITH THE NUCLEOTIDE SITE

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Abstract

Loop 5 (L5) is a conserved loop that projects from the α 2-helix adjacent to the nucleotide site of all kinesin-family motors. L5 is critical to the function of the mitotic kinesin-5 family motors and is the binding site for several kinesin-5 inhibitors that are currently in clinical trials. Its conformational dynamics and its role in motor function are not fully understood. Our previous work using EPR spectroscopy suggested that L5 alters the nucleotide pocket conformation of the kinesin-5 motor Eg5 [1]. EPR spectra of a spin-labeled nucleotide analog bound at the nucleotide site of Eg5 display a highly immobilized component that is absent if L5 is shortened or if the inhibitor STLC is added [1], which X-ray structures suggest stabilizes a L5 conformation pointing away from the nucleotide site. These data, coupled with the proximity of L5 to the nucleotide site suggest L5 could interact with a bound nucleotide, modulating function. Here we use molecular dynamics (MD) simulations of Eg5 to explore the interaction of L5 with the nucleotide site in greater detail. We performed MD simulations in which the L5-domain of the Eg5•ADP X-ray structure was manually deformed via backbone bond rotations. The L5-domain of Eg5 was sufficiently lengthy that portions of L5 could be located in proximity to bound ADP. The MD simulations evolved to thermodynamically stable structures at 300K showing that L5 can interact directly with bound nucleotide with significant impingement on the ribose hydroxyls, consistent with the EPR spectroscopy results. Taken together, these data provide support for the hypothesis that L5 modulates Eg5 function via interaction with the nucleotide-binding site.

Keywords

kinesin-5; nucleotide-analog spin probes; EPR spectroscopy; molecular dynamics simulation

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1. INTRODUCTION

Kinesin-family motors, G-proteins and myosin motors have evolved from a common ancestor protein and share the P-loop, Switch 1 and Switch 2 triphosphate binding domain motif at the nucleotide site [2–4]. All three families are α - β - α proteins with a core β -sheet platform flanked on both sides by α -helices. The Walker-A sequence [5], or P-loop, is a glycine-rich loop (L4) motif located between the core β 3-strand and the flanking α 2-helix and is diagnostic for ATPase proteins. The α 2-helix adjacent to the P-loop in the kinesin-family motors is unique in the superfamily of proteins in that the α -helical repeat is broken two turns from the P-loop into the helix by a surface-exposed element, Loop5 (L5). The α 2-helix then reforms immediately following L5 and extends along the entire surface of the protein. L5 is further of variable length. Homology modeling suggests L5 can be as short as three amino acids in kinesin-14 motors [6, 7], while it is six amino acids in length in the X-ray structure of ncd [8] and 8 amino acids in the structure of kinesin-1 [9]. Conversely, kinesin-5 has the longest known L5-domains, 17 amino acids in length in Eg5 [10].

The X-ray structures of Eg5 with and without bound mitotic inhibitors such as monastrol and S-trityl-L-cysteine (STLC) show that the inhibitors interact with L5 and thus imply that L5 can serve a regulatory function for Eg5 chemomechanics [10–14]. In the X-ray structures with inhibitor, the inhibitor is bound in a pocket formed by the protein surface and L5, with L5 bent away from the nucleotide site. Although X-ray structures with bound inhibitor show small shifts in structural elements, how these would integrate into the observed inhibition remains unresolved. This is in part due to the fact that the L5-domain is in approximately the same conformation in the X-ray structures in both the presence and the absence of inhibitors. Point mutations of the L5-domain, replacement of the Eg5 L5-domain with a kinesin-1 L5-domain, or deletion of 7 residues in the human Eg5 L5 all decrease the ATPase activity of Eg5 [12, 15, 16]. Replacement of the Eg5 L5 with the comparable domain from *Neurospora* eliminates STLC inhibition [17, 18]. All of these observations support a regulatory function for L5.

Most intriguingly, our previous work using nucleotide-analog electron paramagnetic resonance spectroscopy (EPR) probes at the nucleotide site of Eg5 shows two spectral components representing probes bound at the nucleotide site but having different probe mobilities [1]. When the experiments are repeated with the mutant Eg5- Δ L5 protein with a dramatically shortened L5-domain (amino acids 125–131 deleted), only the more mobile component is present in the spectrum. The immobilized component is no longer seen. A similar spectroscopic result is seen with the Eg5•SLADP protein in the presence of STLC. The data raise the possibility that one mechanism of L5 modulation of Eg5 function is via an interaction of L5 with the bound nucleotide, which with EPR probe mobility as the reporter signal, is manifested via a restriction of EPR probe mobility. This would of course require that the L5-domain be very flexible in order to reach over to the nucleotide site. Additional evidence supports this possibility. The x-ray structures of the kinesin-family proteins KIF2 (PDB ID 2GRY [19]), KIF2C (PDB ID 2HEH [6]), KIF22 (PDB ID 3BFN [20]) and kinesin13 (PDB ID 3EDL [21]) all show disordered components of L5, implying flexibility. The effects of L5 mutations on inhibitor binding likewise have been observed to support flexibility [16]. Spectroscopic data measuring the rotational correlation time of a tryptophan residue within L5 have revealed a very high degree of structural flexibility in the absence of drug inhibitors [22], and EPR studies indicate high mobility for probes located within L5 [1].

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 microtubule (MT)-bound states have not been possible. The structure of the MT-bound states must instead be inferred from X-ray structures solved in the absence of MTs. Electron microscopy can resolve secondary structure in a MT-bound state, but not the MT-free states. EPR spectroscopy on the other hand provides lower resolution information. However in the present context, EPR spectroscopy has significant advantages in that it can be used in the presence and absence of MTs in experimental conditions mimicking physiological conditions. EPR spectroscopy can also very easily resolve multiple protein conformations in equilibrium as is the case noted above for Eg5. Computational simulation has proven to be a powerful tool for atomic-level examinations of different conformations of flexible kinesin-family structural elements in X-ray structures [15, 23–27] and their relationship to observations from other experimental techniques. Here we have used the human Eg5•ADP X-ray structure [10] as our starting point for molecular mechanics and molecular dynamics modeling of an Eg5 structure with a deformed L5 loop in order to study possible interactions of L5 with the nucleotide site. Our modeling suggests that the L5 loop in Eg5 is sufficiently long to interact with hydrophobic elements of the bound nucleotide and the adjacent nucleotide pocket, as well as to allow the formation of hydrogen bonds between the negative-charge rich loop and the nucleotide. The simulations imply that L5-nucleotide interaction could play a modulating role in Eg5 function.

2. METHODS

The X-ray structure of human kinesin-5 (Eg5) with bound ADP (PDB ID 1II6, ref [10]) served as the starting point for the simulations. Only the A-chain of the dimeric structure was retained. Structures were visualized and manipulated using the Chimera [28] graphics program. The bond between the backbone N and C α atoms of amino acids 117 or 133, at either end of Loop 5, was removed. The loop was then sequentially maneuvered by ϕ - ψ backbone bond rotations of amino acids 117–133 so that Loop-5 was bent over the ADP moiety in the active site. Six separate starting structures were generated in this manner with Loop 5 coming straight down over the nucleotide or coming down with a twist in a figure-8 motif. After manipulations, the distance between the broken-bond atoms was less than 2Å with approximately the correct bond-angle orientation. The bond was then reestablished using the Chimera function “bond”. For the simulation of the Eg5•ADP/STLC complex, the starting structure was the X-ray structure of the complex (PDB ID 3KEN, ref. [14]). The structure was not modified prior to MD simulation.

Molecular dynamics simulations were performed using the Amber 10 [29] suite of codes. Charges for ADP and STLC were determined by first performing a single-point energy calculation at the Hartree-Fock level of theory using a 6-31G* basis set to obtain electrostatic charges using Gaussian03 [30]. These were then fit to the molecules using the RESP procedure [31]. Van der Waals parameters for Mg²⁺ were taken from [32] as modified in [26]. The tLeap module of Amber 10 was used to add hydrogens to the structure. Histidines were protonated as appropriate for their local environment. Na⁺ counterions were then added to produce overall charge neutrality. A box of TIP3P water molecules [33] was used to solvate the system with a minimum 10Å border between the edge of the box and the closest point on the protein surface. Amber uses a geometric algorithm to place waters. The total size of all simulations after the addition of water molecules was in excess of 55,000 atoms.

The entire protein-water system was energy minimized using 1500 steps of the steepest descents algorithm and a subsequent 1500 steps of the conjugate gradient algorithm. The minimization quickly corrected any incorrect bond lengths and angles resulting from the editing of the structure to modify L5. The minimized system was then gradually heated and maintained at 298K using the Berendsen algorithm [34] for maintenance of temperature via

coupling to an external bath. The particle-mesh Ewald procedure [35–37] with a non-bonded cutoff of 8 Å was used to handle electrostatic interactions. Periodic boundary conditions were employed at constant pressure. MD simulations employed the SHAKE algorithm [38] and a 1 fs time step. All simulations were done for 4 ns. Protein Data Bank format files for all final structures shown in Figs 6–7 and in Figs. S2–S5 (supplementary online material) are available by contacting the authors.

To generate the Eg5-ΔL5 mutant, amino acids 125–131 were deleted from the PDB file of Eg5•ADP (PDB ID 1II6[10]). Backbone ϕ - ψ bonds in the remaining L5 domain were rotated to bring the amino and carboxyl termini of amino acids 124 and 132 within 2 Å at approximately the correct bond angle, and the bond between them reestablished. The structure was then energy minimized as described above and the resulting structure used as the starting point for simulations with the Eg5-ΔL5 mutant. The shortened, mutant L5 was then modified as described above using ϕ - ψ backbone bond rotations to test whether it could interact with ADP at the active site.

High temperature simulations were used to further explore conformations available to L5. These used the generalized Born approach. In order to eliminate crystal forces, the starting structure for the high-temperature simulations was the MD-simulated structure of Eg5•ADP (PDB ID 1II6 [10]) following 5 ns of MD simulation using the particle mesh Ewald algorithm in a box of explicit TIP3P water molecules (described above). The water molecules were removed from the resulting Protein Data Bank format file. For the generalized Born simulation, the structure was then energy minimized using 1000 steps of steepest descent minimization followed by 1000 steps of conjugate gradient minimization, with all atoms except those in Loop5 (amino acids 117–133) restrained by a weak harmonic constraint (1.0 Kcal/mol/Å²). The evolution of the minimized structure was then simulated for 10 ns following gradual heating to 600K. All atoms except those in Loop5 were again restrained by the harmonic constraint. Backbone ω -bonds were constrained to remain in the trans-conformation.

3. RESULTS

Experimental motivation

Figure 1 shows spectra obtained from Eg5 in the presence of the nucleotide-analog spin probe, 2',3'-SLADP. Similar spectra are also shown in [1]. The structure of 2',3'-SLADP is in Fig. S1. The three central peaks, P2–P4, arise primarily from free probe tumbling rapidly in solution. When the EPR probe binds to Eg5, the adjacent protein surface restricts probe motion in the face of thermal agitation. This results in a broadening of the spectrum and the presence of the down-field and up-field components P1 and P5 in the spectra. However, as is clear from the spectrum for Eg5•2',3'-SLADP (red), there is an additional low-field shoulder component denoted by P1* and a corresponding up-field P5* component that are missing in the spectra of Eg5-ΔL5•2',3'-SLADP (blue) and Eg5•2',3'-SLADP in the presence of STLC (cyan). The P1*–P5* component to the spectrum has even greater splitting than that of the P1–P5 components. Greater low-field to high-field splitting in the EPR spectrum implies more restricted mobility of the EPR probe [39]. Spectra similar to that of Eg5-ΔL5•2',3'-SLADP, and missing the second more immobilized P1*–P5* component, have also been obtained with ncd and kinesin-1 [40]. The L5 domain of kinesin-1 is 8 amino acids in length [9]. For ncd it is six amino acids in length [8]. Additional and more complete details of the experimental observations are in [1].

Fig. 2 shows a ribbon diagram of the X-ray structure of Eg5•ADP (PDB ID 1II6, ref. [10]) overlaid with the structure of Eg5•ADP co-crystallized with bound STLC (PDB ID 2WOG, ref. [13]). L5 is colored green and magenta in Eg5•ADP. Magenta is the portion that is

removed in the Eg5- Δ L5 construct. L5 is colored blue in the Eg5•ADP/STLC structure. As is clear, both L5-domains bend away from the nucleotide site containing bound ADP (red). The conformation of the L5-domain of Eg5•ADP/STLC is further stabilized by hydrophobic interactions with STLC (dark gray). The structure of Eg5•ADP co-crystallized with bound monastrol (PDB ID 1X88, ref [12]) shows a similar L5 orientation with monastrol in the same spatial location as STLC.

Summarizing the experimental observations, the P1*–P5* component of the EPR spectrum is seen only for Eg5•2',3'-SLADP. It is not seen for Eg5- Δ L5•2',3'-SLADP, for K•2',3'-SLADP or for ncd•2',3'-SLADP. The latter all contain shortened L5-domains. It is further not seen in the spectrum of Eg5•2',3'-SLADP/STLC. X-ray structures suggest that STLC results in a further stabilization of the L5-domain in a conformation pointing away from the nucleotide site [13, 14]. These observations all suggest that L5 could modulate wild-type Eg5 function via an interaction with the active site. We use MD simulation to investigate this possibility.

Molecular dynamics simulations of the Loop 5 domain

Initial (control) simulations were done with the unmodified x-ray structure of Eg5•ADP (PDB ID 1II6, ref [10]) to examine the inherent stability of the native L5 in the X-ray structure. Fig. 3A, B shows the result of the simulation of Eg5•ADP. Fig. 3A is a ribbon diagram side view of the region of the protein adjacent to L5. Fig. 3B is the view from the top of Fig. 3A. The original X-ray structure is in turquoise. The structure to which it evolves during the MD simulation is in coral. As is clear from the figures, L5 is displaced from ADP at the nucleotide site in the X-ray structure and remains displaced during the MD simulation. Fig. 4A shows the rms deviation of the C α -C α backbone atoms for the evolution of the X-ray structure of Eg5•ADP (red trace). The plot shows a leveling off to a new equilibrium in approximately 2 ns with a maximum rms displacement of slightly less than 3 Å. Fig. 4B (red trace) shows the RMS deviation of L5 (C α -C α atoms, amino acids 117–133) during the same simulation, with a slightly lower RMS deviation, suggesting relatively more perturbation of the entire solvated protein from the X-ray structure. The maximum C α displacement of L5 is 4.5 Å at Tyr 125. Thus there is only a small perturbation of the protein and L5 during the simulation. Most importantly, L5 does not move adjacent to ADP during the simulation. It remains at a location where it does not interact with ADP, as seen in the X-ray structure. A similar simulation was also done using the X-ray structure of the Eg5•ADP/STLC complex as the initial structure. The MD simulation again showed that the X-ray structure was stable on the MD time scale, with L5 bent away from the nucleotide site and not interacting with ADP (data not shown).

An additional simulation was done using the Eg5- Δ L5 construct. Fig. 3A, B show the side and top views of our constructed (Methods) Eg5- Δ L5-domain modified via ϕ – ψ angle backbone rotations to bring it as close as possible to ADP (red trace). It is clear from the figure that the Eg5- Δ L5 construct is simply too short to interact significantly with the ADP moiety at the nucleotide site. The purple trace shows the evolved structure after 4 ns of MD simulation, which has moved away slightly farther from the nucleotide. Fig. 4A (gray trace) gives the rms deviation as a function of MD-simulated time. Again a new equilibrium is reached after approximately 2 ns, with a slightly greater rms value (5 Å) than for the wild-type L5-domain structure. The increased rms value appears to arise from random displacements of loops away from the nucleotide site, and not any components at the nucleotide site. Fig. 4B (gray trace) gives the rms deviation of only the L5 domain during the simulation, which is somewhat larger than observed for the wild-type protein (red trace). However, none of the values suggest extremely large-scale movements during the MD simulation. The maximum C α -C α displacement of the Δ L5-domain is 9.5 Å at Pro 121. This is near the top apex of the loop, which points in different directions at the beginning (red)

and end (purple) of the simulation. Three other attempts at modifying the L5-domain of the Eg5- Δ L5 construct via $\phi - \psi$ angle backbone rotations gave similar results in that the resulting structure did not interact with ADP at the nucleotide site (data not shown). From the molecular dynamics simulations, we conclude that the L5-domain of the Eg5- Δ L5 construct cannot significantly interact with ADP, consistent with the EPR spectra. Furthermore, the L5-domain of the MD simulation of the X-ray structure of Eg5 represents an equilibrium conformation that is compatible with the X-ray structure.

The question remains whether there are other L5 conformations that can interact with ADP, but have not yet been caught in the X-ray database? To investigate this question, L5 was deformed using computer graphical techniques to attempt to maximize any possible interaction with ADP (Methods). Six structures were generated and then analyzed using MD simulation. Fig. 5A, B and C give side, top and end-on (from nucleotide site) views of the L5 and the adjacent region following 4ns MD simulations for five of the deformed L5-domains and the X-ray structure (magenta with the Δ L5 deletion colored in black) again shown for comparison. Figs. 4A,B give the rms deviations ($C\alpha - C\alpha$) from the initial simulation structure as a function of time for the entire protein (Fig. 4A) and for Loop5 (Fig. 4B). Traces for structures for which the intact Loop5 domain has been deformed over the nucleotide-binding site are color-coded to the colors of the structures in Fig. 5. The important observation is that all structures, including the X-ray structure, level off to a new equilibrium structure with a rms deviation value 2–3Å after 2–3 ns of MD simulation. There is somewhat more total relative variability in the RMS deviations of the L5 domain alone. The initial deformations of the L5 domain were based solely on backbone bond rotations. The variability in the RMS deviations is related to the subsequent movements of the L5 domain early in the simulations as intra-protein interactions were forming. However, these are not extremely large-scale movements of L5. Most importantly, the perturbations of the protein in the MD simulations resulting from the modifications of L5 are not causing any significantly increased destabilization of the protein beyond the changes that are seen in the MD simulation using the Eg5•ADP X-ray structure (PDB ID 1II6) as the starting point of the simulation.

Fig. 6 shows higher resolution detail of representative stabilizing forces for the interactions of L5 with the nucleotide and the remainder of the protein for the simulations in which the conformation of L5 was modified. In Fig. 6, there are extensive intra-loop5 interactions in addition to interactions with the α 2- (Thr112-Thr114) and α 3-helices (Arg221). There is additional interaction with the short α -helix at the C-terminus of the β 1-strand (Leu30-Arg33). This includes a hydrophobic stacking of the side-chain of Leu30, the side-chain ring of Trp127, the adenine ring of ADP and the phenyl ring of Phe113 (latter not shown for clarity). Fig. 7 shows an alternative conformation. In addition to intra-loop5 stabilizing interactions, there are interactions with the α 1- (Lys77) and α 2-helices (Thr112), the β 3-strand (Arg24), with the side-chain ring of Trp127 located in a pocket formed by the hydrophobic portions of the side-chains of Asp130, Ala133, Arg119 and Thr112. The simulations in Figs. 6 and 7 were chosen randomly as representative. Other deformed conformations of L5 obtained from MD simulations with different initial deformations of L5 are shown in Figs. S2–S5 (Supplementary online material). In general, these conformations show interactions of the short α -helix at the C-terminus of the β 1-strand (Leu30-Arg 33) and the α 1-helix (Lys77) with L5. Interestingly, a recently solved X-ray crystal structure of Eg5 in the presence of AMPPNP (3HQD) shows some movement of these elements relative to their position in other structures (1II6, and 3KEN) [10, 41], indicating that some flexibility in these elements may accommodate different stable conformations of L5. As a further test of the stability of the deformed L5 conformations, the simulations were extended to 24 ns total simulation time. Compared to the L5 position after 4 ns (Fig. 4), the rms displacements of L5 ($C\alpha - C\alpha$) after 24 ns were 3.1Å (red), 2.2Å (orange), 2.3Å (green), 2.1Å (cyan), 3.5Å

(blue), 2.8Å (magenta). All L5 domains remained projected over the nucleotide with little change in position during the additional 20 ns of simulation.

The above simulations demonstrate the MD time-scale stability of L5 conformations that impinge upon the nucleotide site. The L5 conformations were manually formed. A remaining question is whether L5 can actually deform into such impinging conformations in the face of thermal displacement. To address this question, we used the generalized Born approach for high-temperature MD simulations (details described in Methods). Higher temperature allows the simulation to more rapidly cross energy barriers than would be possible at physiological temperatures. Thus the feasible conformational space may thus be more rapidly sampled. Figure 8 shows the simulation approach applied to the Eg5•ADP X-ray structure (PDB ID 1II6, see Methods for details). Figure 8 shows the simulated location of L5 every 600ps during a 10 ns simulation run at 600K. Fig. 8A shows a “side” view with L5 at the top of the frame and ADP (standard atom colors) on the left-hand side of the frame. Fig. 8B shows the same structures from a point of view that looks down on L5 with the α 2-helix underneath L5 and ADP partly obscured by L5 at the left-hand side. The EPR spectroscopy observation that raised the possibility of interaction between L5 and the nucleotide was from observations of 2',3'-SLADP bound at the nucleotide site of Eg5 and the hinderance of the mobility the pyrrolidine spin-ring moiety. The spin ring is bridged by a carbonyl linkage to O2'- or the O3'-position the ribose ring of ADP. The ring plus leash is approximately 5.5Å in length and the pyrrolidine ring is 6.6Å across at its widest (spanning across the methyl groups). A diagram of SLADP with spin-ring moiety is in Fig. 1, ref. [40]. The important observation is that approximately half of the L5 orientations seen in Fig. 8 would provide additional restrictions on the motion of the attached spin probe beyond that of the native nucleotide site itself. This is again compatible with the experimental observation that the EPR probes bound to Eg5 see additional restriction of mobility.

4. DISCUSSION

Kinesin-family motors contain an unusual Loop 5 structure that breaks the α 2-helix two-turns from the P-loop and projects out from the α 2-helix into the aqueous environment. L5 is of variable length in different kinesin-family motors. L5 is surprisingly absent from kinesin's evolutionary cousins, the myosin motors and the G-proteins [3], suggesting kinesin motor-specific function(s). The function of L5 remains unresolved. Interest in L5 function has been heightened by X-ray crystallography observations that L5 is involved in the binding of small-molecule, allosteric inhibitors of Eg5 [11, 12, 14, 42]. The specificity of the allosteric inhibitors to kinesin-5 motors and the obligatory involvement of Eg5 in mitosis offer the potential for the development of novel anti-cancer therapies.

In the x-ray structures of all kinesin-family motors with a nucleotide or a nucleotide analog at the active site, the ribose hydroxyls point out into the aqueous environment. This would place the spin moiety of 2',3'-SLADP (Fig. S1) also projecting into the aqueous environment where it would be in a position to interact with a sufficiently long L5. The presence of a more immobilized component in the experimentally observed EPR spectrum of Eg5•2',3'-SLADP, coupled with the absence of the more immobilized component in a species with a shortened L5-domain, or an L5 domain stabilized in a conformation away from the nucleotide site as indicated by the X-ray structure of Eg5•2',3'-SLADP/STLC, initially suggested a direct interaction of L5 with the bound nucleotide. Supporting this idea, the MD simulations in this work demonstrate that the 18 amino-acid long L5-domain is sufficient in length to interact with the nucleotide. Furthermore we find that the shortened L5-domain of Eg5- Δ L5 does not interact significantly with the nucleotide in the MD simulations. Multiple attempts at deforming the Δ L5-domain into a configuration that would stably interact with

the nucleotide failed. Interaction of L5 with the nucleotide site is further supported by studies suggesting that mutations of L5 perturb the Eg5 kinetic cycle [1, 12, 15–18, 22].

Surprisingly, the length of the L5-domain allowed for multiple conformations of L5 that formed new stable equilibrium conformations on the MD time scale (Figs 6–7 and Figs. S2–S5). Depending upon the specific simulation, inter-protein interactions were seen between L5 and the α 1-, α 2- and α 3-helices, the β 1-strand, and the short α -helix at the C-terminus of the β 1-strand. To again emphasize the size of L5, we note that the latter short α -helix is located on the opposite side of the bound ADP adenine ring from the point of projection of L5 out of the α 2-helix. Another unusual feature of L5 is the presence of the tryptophan residue, Trp127. In Figs. 6 and 7 it is involved in extensive hydrophobic interactions helping to stabilize the new conformation of L5. However, in other MD-simulated conformations, it is exposed to solvent. Although we are seeing multiple equilibrium conformations of L5 interacting with the nucleotide, we cannot rule out the possibility that given sufficient time, the intra-protein energetics would result in the multiple conformations evolving into a smaller subset, or a single preferred interacting conformation. Existing data from Maliga, et al. [22] indicate that W127 is highly dynamic in the absence of bound drug, and even exhibits some dynamics in the presence of drug. These may be short-lived conformations that nonetheless have a profound effect on Eg5 kinetic properties.

A significant observation is that stable interactions with the nucleotide site were formed for all six of our initial conditions with the intact L5 domain deformed over the nucleotide site. No simulation failed to interact with the nucleotide. The reason is that the L5 domain is sufficiently lengthy that such interactions are readily feasible and to be expected. However, the control MD simulations using the X-ray structure as the starting point showed an additional equilibrium position for the L5 domain with it pointing away from the nucleotide-site, not interacting with bound nucleotide, and stabilized primarily by W127 interacting with other hydrophobic parts of the protein. These observations are not in conflict. They provide further support for the hypothesis that there are multiple equilibrium configurations for L5, each representing a local free energy minimum on a global free energy surface, with X-ray structures to date having only captured one of the conformations of L5. These nucleotide-interacting conformations of L5 need not necessarily be long-lived; explaining why the MD/EPR spectroscopy approach used here has identified them while crystallography has yet to capture them. The simulations with Eg5- Δ L5 provide further support. Here unlike native Eg5, the deformed L5 of Eg5- Δ L5 that served as the initial condition for the simulation was too short to form any new intra-protein contacts to stabilize an interaction with the nucleotide. Thus the deformed conformations were not stable.

X-ray structures are fixed snapshots of a multi-conformation process. MD based on initial X-ray structures and EPR spectroscopy data has revealed functionally relevant conformations of kinesin motors in the past, many years before those conformations were revealed using experimental techniques. Initial X-ray structures from the year 1996 showed a single conformation of Switch 1, pulled away from the nucleotide opening the nucleotide pocket [8, 9, 43]. MD simulations performed in 2001 on the Switch 1 domain at the nucleotide-site of kinesin-family motors yielded multiple, stable, equilibrium conformations, just as we see here for the Eg5 L5. In particular, these simulations revealed a stable conformation with the Switch 1 domain adjacent to the nucleotide, closing the nucleotide-pocket [26]. Only very recently in 2010, an X-ray crystal structure was solved for a kinesin family member having a similar closed Switch 1 conformation [41].

Relationship to other work

A number of differing functions have been proposed for L5. In the X-ray structures of kinesin-family motors, the adenine ring is bound in a hydrophobic slot formed on one side

by the adenine-binding loop frequently termed N4 [4]. This includes the ring of Pro27 and the propyl-group of Arg26 in Eg5. The “lid” of the pocket on the solvent side is conserved as a histidine, tyrosine or phenylalanine in kinesin-family motors (Phe113 in Eg5). Thus other than hydrophobic stacking, there is little to hold intact the adenine-binding pocket of kinesin-family motors. MD simulations originally showed this could result in a concurrent rotation of the amino-acid side-chain lid ring and adenine ring out of the binding pocket of *ncd*•ADP or kinesin•ADP. The rotation of the two stacked rings was stopped by steric interference from amino acid side chains at the base of L5. This led to our original suggestion that L5 served simply as a “bumper stop” to modulate nucleotide binding [26]. In myosins the lid is instead the hydrophobic portion of long, charged side chains, with the resultant salt-bridge providing additional stabilization. In G-proteins, there are additional ionic and hydrogen-bonding interactions to the bound guanine ring to help stabilize the bound nucleotide in the binding site. This may help to explain why L5 is missing in the latter two protein families.

A number of lines of evidence have suggested that an interaction between L5 and the adjacent α 3-helix may likewise be involved in modulating motor function. A direct interaction was first observed in the X-ray structure of the non-motile kinesin-10 motor NOD [44], with the first 6 amino acids of L5 swinging away from the nucleotide site and packing against the α 3-helix. EM reconstructions of microtubule-bound *Drosophila* kinesin-5 motor KLP61F likewise indicated that L5 undergoes a microtubule-binding dependent interaction with the α 3-helix that was postulated to be involved in the transition between force-generating and diffusional modes of MT binding [45]. Spectroscopic and biochemical data have additionally linked L5 to the force-generating cycle through changes in the conformation of the neck-linker [1, 15, 46] that have been proposed to be modulated via interaction with the α 3-helix and concomitant perturbations of Switch 1 by the α 3-helix [15]. We observed only minimal interaction of L5 with the α 3-helix in our simulations (a single interaction with Arg221 in two of the six simulations). However, our observations and the above observations are not in conflict. Our distortions of L5 to generate the initial conditions for the MD simulations were biased to emphasize interactions with the nucleotide site. L5 is clearly of sufficient length to generate interactions with the α 3-helix that are even more extensive than are currently present in the X-ray and EM databases.

In summary, as previously noted, mutations of L5 have been implicated in perturbations of the Eg5 kinetic cycle [1, 12, 16–18, 22]. Here we have suggested another possible function for L5 in kinesin-5 motors, modulation of nucleotide binding via direct physical interaction with the nucleotide site. L5 is of surprisingly variable length over the breadth of the kinesin-family motors. There is likewise minimal sequence conservation. Considering the structural and functional results *in toto*, it seems plausible that L5 may play multiple functions that are dependent on the specific kinesin-family motor in question.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Loop5 in Eg5 projects from the protein adjacent to the nucleotide site.
- Loop5 modulates Eg5 function by unresolved mechanisms.
- Spectroscopy has suggested that loop5 may interact with the nucleotide site.
- MD simulations are used to quantitatively test the interaction hypothesis.
- The simulations show loop5 can interact extensively with the nucleotide site.

Figure 1.

X-band EPR spectra of 2',3'-SLADP bound to Eg5 (red), Eg5-ΔL5 (blue) and Eg5 in the presence of the inhibitor, STLC (cyan). The horizontal axis is magnetic field. The vertical axis is the derivative of absorbance. The spectra are 9.0 mT wide with center field at 350 mT. The figure is similar a previously published figure [1] and is present to motivate the computational modeling.

Figure 2.

An overlay of ribbon diagrams of the structures of Eg5•ADP [10] and Eg5•ADP with bound STLc [13]. Eg5•ADP is colored coral except for L5, which is colored green and magenta. The magenta segment is the portion of L5 that is removed in the Eg5-ΔL5 construct. Eg5•ADP with bound STLc is colored turquoise, except for L5, which is blue. STLc is gray. ADP is red.

Figure 3A

Figure 3B

Figure 3.

A. The X-ray structure of Eg5•ADP (turquoise), Eg5•ADP following 4ns of MD simulation (coral), the constructed structure of Eg5-ΔL5•ADP distorted to be as close as possible to ADP at the nucleotide site (red), and the structure of Eg5-ΔL5•ADP following 4 ns of MD simulation (purple) are shown as ribbon diagrams. The structures were superimposed using a least-squares distance minimization of the P-loop C α atoms. ADP from the X-ray structure of Eg5•ADP is in dark gray. Only the regions of the proteins adjacent to the nucleotide site and L5 are shown. Fig. 3A is oriented from the side with the L5 at the top of the panel. **B.** Same a panel A, but looking down from the top of panel A.

Figure 4B

Figure 4.

A. The RMS deviation of the MD-simulated structures as a function of time. Color coding: Eg5•ADP (red), Eg5-ΔL5•ADP (gray). The cyan, green, orange, blue and magenta plots are color-coded to the structures in Fig. 5. **B.** RMS deviation of only the Loop5 domain during the simulation. Color-coding is as in panel A.

Figure 5A

Figure 5B

Figure 5C

Figure 5.

The structures of five different deformations of L5 in the Eg5•ADP structure (blue, cyan, red, green, orange) interacting with ADP bound at the nucleotide site (dark gray) following 4ns of MD simulation. The structures were superimposed using a least-squares distance minimization of the P-loop C α atoms. The structure of Eg5•ADP (magenta with the domain deleted for Eg5- Δ L5 in black) is shown for comparison. Fig. 5A is oriented from the side with L5 at the top of the panel. Fig. 5B looks down from the top of panel A. Fig. 5C looks from a direction facing the bound ADP.

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Figure 7.
Ribbon representation of the interactions (ionic, hydrogen bonds, and hydrophobic) that result in the forces stabilizing the location of Loop5 (red) in one observed conformation from the MD simulations.

Figure 8. Ribbon diagrams of a superposition of Loop 5 conformations from high temperature molecular dynamics simulation. Individual frames are from a 10 ns simulation with frames separated by 600 ps.