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Nucleotide-Induced Flexibility Change in Neck Linkers of Dimeric Kinesin as Detected by Distance Measurements Using Spin-Labeling EPR

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Using dipolar continuous-wave and pulsed electron paramagnetic resonance methods, we have determined the distribution of the distances between two spin labels placed on the middle of each of the neck linkers of dimeric kinesin. In the absence of microtubules, the distance was centered at 3.3 nm, but displayed a broad distribution with a width of 2.7 nm. This broad distribution implies that the linkers are random coils and extend well beyond the 2.5-nm distance expected of crystal structures. In the presence of microtubules, two linker populations were found: one similar to that observed in the absence of microtubules (a broad distribution centered at 3.3 nm), and the second population with a narrower distribution centered at 1.3–2.5 nm. In the absence of nucleotide but in the presence of microtubules, ~40% of the linkers were at a distance centered at 1.9 nm with a 1.2-nm width; the remaining fraction was at 3.3 nm, as before. This suggests that neck linkers exhibit dynamics covering a wide distance range between 1.0 and 5.0 nm. In the presence of ATP analogs adenosine 5′-(β,γ-imido)triphosphate and adenosine 5′-(γ-thio)triphosphate, 40–50% of the spins showed a very narrow distribution centered at 1.6 nm, with a width of 0.4–0.5 nm. The remaining population displayed the broad 3.3-nm distribution. Under these conditions, a large fraction of linkers are docked firmly onto a motor core or microtubule, while the remainder is disordered.

We propose that large nucleotide-dependent flexibility changes in the linkers contribute to the directional bias of the kinesin molecule stepping 8 nm along the microtubule.

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Keywords: kinesin; neck linker; flexibility; interspin distance; EPR

Introduction

Active transport is mediated by the interaction of kinesin with microtubules[1,2] in a wide variety of cellular processes such as organelle movement, cell division, and fast axonal transport.[3] Conventional kinesin is a highly processive motor that converts the chemical energy of adenosine triphosphate (ATP) into a unidirectional movement along microtubule tracks. The molecular details for kinesin motion and processivity have been studied extensively using genetic, biochemical, structural, and single-molecule analyses, as described below. A recent fluorescence imaging study suggested that kinesin walks hand-over-hand.[4] This processive walking motion is believed to depend on the coordination between the ATPase cycle of two motor domains and their neck linkers. The neck linker is thought to be one of the essential elements of kinesin movement. This region (15 amino acids and ~3 nm long) connects the C-terminus of the kinesin

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Abbreviations used: cryo-EM, cryo-electron microscopy; EPR, electron paramagnetic resonance; FRET, fluorescence resonance energy transfer; AMPPNP, adenosine 5′-(β,γ-imido)triphosphate; CW, continuous wave; DEER, double electron–electron resonance; PDB, Protein Data Bank; MSL, 4-maleimido-2,2,6,6-tetramethyl-1-piperidinoxy; ATPγS, adenosine 5′-(γ-thio)triphosphate; NN, no-nucleotide; MTSSL, (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)methane thiosulfonate.

catalytic core to the helical region in the neck; in crystal structures, it is docked on a surface of the core to form a β-sheet.[5] The neck stabilizes the dimeric structure of kinesin and is followed by the tail domain that binds a cargo at its distal end.

Mutational analysis showed that the catalytic core is sufficient for microtubule binding and ATPase activity; however, the kinesin neck linker functions as a mechanical amplifier and determines the direction of motion.[6–9] Cryo-electron microscopy (cryo-EM), electron paramagnetic resonance (EPR), and fluorescence resonance energy transfer (FRET) studies of Rice *et al.* have shown that ATP induces nucleotide-dependent conformational changes (i.e., 'disordered' and 'docked' forms of the neck linker) in monomeric kinesin when bound to microtubules.[10] Nucleotide-dependent conformational changes in the neck linker were also detected by fluorescence polarization microscopy.[11] This conformational change has been suggested to be responsible for both the direction of kinesin motion and the processive movement by dimeric kinesin. Cross-linking the neck linker by disufide bonding abolished kinesin motility.[12]

How the kinesin dimer spans the 8 nm between adjacent tubulin binding sites[13,14] when, in the crystal structure of dimeric kinesin, the two motor domains are only 5 nm apart has been puzzling.[5] The conformations of dimeric kinesin bound to the microtubule have been resolved by cryo-EM and single-molecule fluorescence polarization or FRET microscopy.[11,15–17] These studies showed that in the microtubule-bound motor domains, the neck linkers have opposite directionality and are ordered in the presence of adenosine 5′-(β,γ-imido)triphosphate (AMPPNP), but disordered in the absence of nucleotide. In the presence of ADP, the monomeric kinesin binds in a disordered configuration, but the disorder is reduced by the partner motor domain in dimeric kinesin. In our previous report, we used site-directed spin-labeling EPR spectroscopy to determine the conformations of the neck linker in dimeric kinesin in the presence of microtubules.[18] The neck linker was found to be in equilibrium between the mobile (or undocked) conformation and the immobile (or docked) conformation. The equilibrium did not depend on the nucleotide states. The absence of nucleotide-induced changes in dimeric kinesin was at odds with changes observed in monomeric kinesin.[10,19,20] The neck linker proximity of dimeric kinesin was monitored with fluorescence quenching caused by the dimerization of two fluorescence labels attached to each of the neck linkers.[21] The results suggested that the opening and reclosing of neck linkers occur after ATP has been added. Here we have measured the distance between two spin labels in the middle of the linkers of dimeric kinesin by dipolar continuous-wave (CW) EPR[22–25] and pulsed double electron–electron resonance (DEER) EPR.[23,26,27] We found that the distance distributions of kinesin microtubules changed remarkably with the binding of different nucleotides to kinesin on microtubules. This supports the hypothesis that the switch in flexibility, or the undocked–docked cycle of the linkers, is closely coupled to force or bias generation during the movement of the motor domain on the microtubule.

Results

Spin labels in two neck linkers of the kinesin dimer are distant in the microtubule-free state

We have measured dipolar interactions between spin labels at Cys332 (red balls) in the middle of the neck linkers (peptides indicated in yellow, ~15 amino acids and ~3 nm long) of dimeric kinesin (Fig. S1). Comparison with the crystallographic model[5] of dimeric kinesin [Protein Data Bank (PDB) ID 3KIN] was accomplished at the 4-maleimido-2,2,6,6-tetramethyl-1-piperidinoxy (MSL)-modified side-chain level rather than at the C^{α}–C^{α} level of Cys332. To fully account for spin-label size and orientation with respect to the protein, we modeled the probes using the molecular modeling described in Materials and Methods. Minimum energy structures were first found by Monte Carlo methods (Fig. S2a), followed by molecular dynamics simulations (Fig. S2b) of the doubly labeled molecule.[26] In the Monte Carlo methods, the probability of each combination of conformers was weighed according to the Boltzmann distribution of their relative energies. The distance between nitroxide oxygen atoms was then calculated for the 90,000 lowest-energy conformers of the two spin labels (Fig. S2a). The molecular dynamics trajectory was calculated at 300 K using the lowest-energy conformer from Monte Carlo methods as starting structure (Fig. S2b). The distance estimated from this modeling was 2.0±0.2 nm, which is within the range of sensitivity for both EPR techniques.

Comparison of the doubly labeled EPR spectrum for the microtubule-free kinesin dimer with the spectrum of monomeric kinesin revealed no evidence of spectral line broadening. This suggests that the distance between the spin labels in the dimer is longer than 2.5 nm, the limit of the dipolar CW-EPR method. The nucleotides AMPPNP and ADP did not change the spectrum (Fig. 1a). The absence of broadening is not due to substoichiometric labeling (>80%), as >80% of the signal originates from the doubly labeled sample, and 0.5 G broadening, if present, would be readily observed. More likely, the neck linkers are flexible, and Brownian motion separates the motor cores further than in the crystal structure.

To measure these longer distances, we employed a four-pulse DEER method that is sensitive to distances from 2 to 7 nm. The spectra of kinesin dimers in the absence of microtubules are shown in Fig. 2a. The distance population that gives rise to these spectra was obtained by assuming a Gaussian distribution and by calculating the resulting DEER spectra. The number of Gaussians was increased from 1 to 4, and statistical *F*-test was applied to