Kar3Vik1 Mechanochemistry Is Inhibited by Mutation or Deletion of the C Terminus of the Vik1 Subunit**

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Background: Kinesin motility is driven, in part, by structural rearrangements of the motor domain N and C termini. Force production by kinesins has been linked to structural rearrangements of the N and C termini of their motor domain upon nucleotide binding. In recent crystal structures, the Kar3-associated protein Vik1 shows unexpected homology to these conformational states even though it lacks a nucleotide-binding site. This conservation defers a degree of commonality in the function of the N- and C-terminal regions during the mechanochemical cycle of all kinesins and kinesin-related proteins. We tested this inference by examining the functional effects on Kar3Vik1 of mutating or deleting residues in Vik1 that are involved in stabilizing the C terminus against the core and N terminus of the Vik1 motor homology domain (MHD). Point mutations at two moderately conserved residues near the Vik1 C terminus impaired microtubule gliding and microtubule-stimulated ATP turnover by Kar3Vik1. Deletion of the seven C-terminal residues inhibited Kar3Vik1 motility much more drastically. Interestingly, none of the point mutants seemed to perturb the ability of Kar3Vik1 to bind microtubules, whereas the C-terminal truncation mutant did. Molecular dynamics simulations of these C-terminal mutants showed distinct root mean square fluctuations in the N-terminal region of the Vik1 MHD that connect it to Kar3. Here, the degree of motion in the N-terminal portion of Vik1 highly correlated with that in the C terminus. These observations suggest that the N and C termini of the Vik1 MHD form a discrete folding motif that is part of a communication pathway to the nucleotide-binding site of Kar3.

Kinesins are microtubule-based motor proteins with roles in intracellular movement that include vesicle and organelle trafficking and chromosome segregation (1). They are typically assembled from two identical subunits whose microtubule interactions and force production cycles involve conformational changes in deformable regions of the motor domain (MD)³ in response to nucleotide binding (2). These regions include the short segments at the N and C termini of the motor domain (3–7). In microtubule plus-end-directed kinesins such as kinesin-1, these form the “cover strand” and “neck linker,” and in the minus-end-directed kinesin-14, they form the “neck” and “neck mimic,” respectively. Docking and undocking of residues near the C terminus of the motor domain correlate with ATP turnover and force production in both plus- and minus-end-directed kinesins (see Fig. 1, A and B) (6–12). In processive kinesins, mechanical strain transmitted through the neck linkers of adjoined motor subunits is thought to mediate gating of the chemical cycles of each subunit and thereby synchronize their microtubule interactions and stepping events (3, 13–17). Although evolution has edited these structural elements to enable specific transport tasks, recent studies indicate that certain design principles have been preserved across different kinesin family members (18). Crystallographic and biochemical studies of the Kar3 kinesin-associated protein Vik1 indicate that this theme may extend beyond the accepted definition of a kinesin motor protein (19).

Kar3 is a kinesin-14 found in most Hemiascomycetes fungi. As a heterodimeric complex with Vik1, it is involved in organizing and bundling mitotic and meiotic spindles through its microtubule cross-linking and sliding activity (20–23). The Vik1 subunit regulates the localization and function of Kar3 via a canonical kinesin motor domain fold that can bind microtubules, but not ATP (24). It has recently been shown that the N-terminal neck region of the Vik1 motor homology domain (MHD) is important to the mechanochemical function of the Kar3Vik1 motor assembly (19). Not only can the neck adopt different conformations that resemble those of kinesin-14 motors during their powerstroke (see Fig. 1C), but perturbation of Vik1 neck rotation impedes the gliding and ATPase activity of Kar3Vik1 complexes (4, 19). This implies that the Vik1 neck is an important structural element of Kar3Vik1 motors and that

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³ The abbreviations used are: MD, motor domain; MHD, motor homology domain; C. glabrata; KCBP, kinesin-like calmodulin-binding protein; CC, coiled coil; N, neck; AMPNP, adenylyl imidodiphosphate; PDB, Protein Data Bank; r.m.s.f., root mean square fluctuation; PCA, principal component analysis.
it transitions between states that either enable or block microtubule interactions and/or ATP binding and turnover in Kar3.

Another commonality between Vik1 and bona fide kinesin-14 motors involves the neck mimic at the end of helix H9251. In recent crystal structures of Candida glabrata (Cg) Vik1, this element docked against the core of the MHD and formed a small two-stranded β-sheet with the neck-core junction (Fig. 1C) (19). Both Ncd and the kinesin-like calmodulin-binding protein (KCBP), two well studied kinesin-14 motors, form a similar structure involving the N and C termini of their motor domains (9, 10). In Ncd, the neck mimic is 36 residues long and contains a high proportion of positively charged amino acids that may interact with the motor core or mediate motor domain binding to microtubules by charge-charge interactions. Replacement or deletion of these residues dramatically changes the microtubule binding and motile function of Ncd, suggesting...
that the neck mimic is an important part of the force-generating apparatus in kinesin-14 motors (11). Although incapable of force generation, perhaps Vik1 uses its C-terminal neck mimic in combination with its N-terminal neck element during other key intermediate states of the Kar3Vik1 mechachemical cycle (25).

We examined the importance of the neck mimic of Vik1 in the mechachemical activity of the Kar3Vik1 motor assembly by mutating or deleting the neck mimic altogether (see Fig. 2A). Mutant CgVik1 constructs consisting of the MHD and a portion of the coiled-coil-forming stalk region were co-purified with a comparable construct of WT CgKar3 to yield mutant heterodimeric motors (see Fig. 3) (19). All of the mutants showed a marked decrease in the number of continuously gliding microtubules and a reduction in microtubule-gliding velocity relative to the WT motor. Microtubule-activated ATPase activity assays of Kar3Vik1 motors showed that mutations at neck mimic residues Ile578 and Asn580 reduced the ATP turnover in Kar3 by at least 3-fold. The largest defects were associated with mutants that strengthened neck mimic docking through Ile578 or that disrupted interactions important for neck isomerization. Molecular dynamics simulations of mutant versions of the CgVik1 MHD x-ray crystal structure showed clear differences in the neck and neck mimic conformations compared with the WT CgVik1 MHD construct. They also provided new insights into interaction networks connecting the neck and neck mimic, which may be critical for transmitting nucleotide binding information from Kar3. This work suggests that neck rotation and neck mimic docking are part of the communication mechanism between Kar3 and Vik1. It also supports previous observations in other kinesins that implicate the neck and neck mimic modules in forming a functional subdomain whose isomerizations underlie discrete mechanical states of the motor.

**Experimental Procedures**

*Mutation and Protein Expression and Purification—Full-length Kar3 and Vik1 sequences were PCR-amplified from *C. glabrata* genomic DNA (ATCC 2001D-5) and ligated into pCR2.1®-TOPO® (Invitrogen). Site-directed mutagenesis of CgVik1 was performed using QuikChange (Stratagene). Each mutant was coexpressed and co-purified with WT CgKar3 as a truncated heterodimeric CgKar3Vik1 complex as described previously (19). The truncated WT CgKar3 construct comprised a portion of the central coiled-coil-forming section, the neck, and the motor domain (CgKar3-CC+N+M) and was cloned into pET24d to yield Met198–Asn692 of CgKar3 when overexpressed. The WT and mutant CgVik1 constructs comprised a portion of the central coiled-coil-forming section, the neck, and the MHD (CgVik1-CC+N+MHD). These were subcloned into a modified version of pET16b (Novagen) in which the Factor Xa cleavage site was replaced with a recombinant tobacco etch virus protease cleavage site, which yielded MGH10 SSGRENLYFGQHM-Asp152-Lys584. The CgVik1A578–584 mutant was generated by subcloning the corresponding section of the CgVik1-CC+N+MHD construct into the same pET16b vector as the point mutants and is expressed as MGH10 SSGRENLYFGQHM-Asp152–Phe577.

All plasmids were transformed into *Escherichia coli* BL21-CodonPlus(DE3)-RIL cells (Stratagene) for protein expression in LB broth. Cells cotransformed with pET16b encoding CgVik1-CC+N+MHD and pET24d encoding CgKar3-CC+N+MD were grown in LB medium supplemented with 50 µg/ml kanamycin, 50 µg/ml ampicillin, and 50 µg/ml chloramphenicol to OD600 ~0.8 and then induced with 1.0 mM isopropyl β-D-thiogalactopyranoside. After continued growth at 20 °C for 16 h, cells were lysed by sonication, and the dimer was purified by nickel-nitritolactric acid affinity chromatography, followed by gel filtration on a HiLoad Superdex 200 26/60 column (GE Healthcare) as described previously (19). All proteins were concentrated and flash-frozen in liquid nitrogen for storage (24). CD spectra of all point or truncation mutants were collected using a Chirascan CD spectrometer (Applied Photophysics Ltd., Leatherhead, Surrey, United Kingdom) to assess for changes in their structure and stability relative to the WT. All samples were dialyzed in 20 mM HEPES (pH 7.2), 150 mM NaCl, 0.1 mM MgCl₂, 1 mM tris(2-carboxyethyl)phosphine, 0.2 mM ATP, 2.5% sucrose prior to CD analysis. For each sample, six replicate CD scans were collected. Preliminary data processing and reference subtraction were performed with Chirascan ProViewer software.

**Analysis of Kinesin Motility—**Motility assays were performed as described previously (19). WT and mutant Kar3Vik1 complexes were clarified by centrifugation before each experiment, and their protein concentrations were determined by measuring the absorbance at 280 nm. Taxol-stabilized rhodamine-labeled microtubules were assembled by mixing rhodamine-labeled bovine tubulin (Cytoskeleton Inc.) with unlabeled bovine brain tubulin at a molar ratio of 1:4. This mixture was polymerized, centrifuged, and resuspended in BRB80 buffer (80 mM PIPES (pH 6.8; KOH), 1 mM MgCl₂, and 1 mM EGTA) supplemented with 40 µM Taxol. Perfusion chambers were constructed by sandwiching 22 × 60-mm and 22 × 22-mm glass coverslips together using double-sided tape.

For each motility experiment, anti-polyhistidine antibodies (Fisher) were used to attach the kinesins to the interior glass surface of the perfusion chamber, which was then blocked with 5 mg/ml BSA. The concentration of Kar3Vik1 motors added to the chambers ranged from 0.4 to 2 µM to generate fields with similar densities of surface-bound microtubules for WT and mutant versions of Kar3Vik1. Microtubules were shedded to generate short filaments and were added to the perfusion chambers at a concentration of 2 µM to form Kar3Vik1-microtubule complexes. Microtubule binding to kinesins was stimulated by inclusion of 1.5 mM MgAMPPNP in oxygen scavenger buffer (1× BRB80 buffer, 1.5 mM MgAc, 1 mg/ml BSA, 200 µg/ml glucose oxidase, 175 µg/ml catalase, 25 mM glucose, 2 mM β-mercaptoethanol, and 40 µM Taxol). After a 5-min incubation, the perfusion chamber was rinsed with oxygen scavenger buffer. Microtubule gliding was initiated by infusing 1.5 mM MgATP in an ATP regeneration system (0.3 µg/µl phosphocreatine kinase and 2 mM phosphocreatine in oxygen scavenger buffer) (26) that included either 0.4–0.8 µM additional Kar3Vik1 or no additional motor. Images were captured at a constant temperature of 25 °C on an Olympus IX-81 inverted microscope with both spinning disc confocal and total internal
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reflection fluorescence imaging capabilities (Quorum Technologies Inc.). Quorum Wave FX Metamorph software was used to process the images and compile them into movies. Microtubule movement was tracked using Image-Pro Plus 6 (Media Cybernetics Inc.).

Only those microtubules that were between 5 and 10 μm long and moved continuously within the entire time frame of the video were tracked for the motility assay data in Table 1. To calculate the percentage of gliding microtubules in Fig. 4, the number of 2–10-μm-long gliding microtubules at each time point was divided by the total number of microtubules visible in the viewing area at the beginning of the video.

Steady-state ATPase Activity—The ATPase kinetics of the WT and mutant Kar3Vik1 motors were determined at 25 °C using a pyruvate kinase/lactate dehydrogenase-coupled NADH oxidation reaction as described previously (25, 27). Data were collected from at least three independent experiments and analyzed, and the plots represent the average of these experiments. Both microtubule and ATP concentration-dependent data were fit to the Michaelis-Menten equation below (Equation 1). For ATP-dependent experiments, the MgATP concentration was varied (0–2 mM) with microtubules held constant between 3 and 5 μM for different mutants.

\[ V = \frac{V_{max} \cdot [S]}{K_m + [S]} \]  

(Eq. 1)

where \( S \) is the substrate concentration (tubulin heterodimer or MgATP), \( V_{max} \) is the maximal rate of steady-state ATP turnover, and \( K_m \) is the steady-state Michaelis constant for microtubules or ATP and represents the concentration needed to provide one-half the maximal velocity.

Molecular Dynamics Simulations—Chain A of the Protein Data Bank (PDB) CgVik1 structure 4GKQ (19) was used as the starting model for all molecular dynamics simulations because it was the most complete of the CgVik1 models. Modeler was used to fill in missing residues Gly299, Phe366–Lys371, Pro403 Asn404, Asn433–Asp435, Asn443–Glu451, Ile573–Gln489, Thr532–Asn535, Gly583, and Lys584 (28). Coordinates of some of the neck residues changed during this process, so the original neck structure (residues 300–322) was grafted onto the structure prepared by Modeler. Mutations (I578F, I578A, N580D, N580K, and N580A) and deletion of the neck mimic (C-terminal residues Ile578–Lys584) were performed with Coot to generate the final models for molecular dynamics simulations (29). All molecular dynamics simulations were performed using the program GROMACS (Version 4.5.5) with the CHARMM27 force field (30, 31). Prior to the full-scale molecular dynamics simulations, all structures were solvated in a box of waters using the TIP3P water model with a 1-nm distance between the protein and the box faces (32). The structures were energy-minimized using the steepest descents algorithm with a tolerance (maximal force) of 100 kJ/mol/nm. This was followed by two 20-ps position-restrained molecular dynamics simulations to relax the solvent around the protein. The first used the NVT (moles, volume, and temperature are conserved) ensemble, and the second used the NPT (moles, pressure, and temperature are conserved) ensemble. All molecular dynamics simulations were performed at a temperature of 298 K and used V-rescale temperature coupling, and the NPT simulations used Parrinello-Rahman pressure coupling. Electrostatics were treated with the particle mesh Ewald method with a 1.1-nm cutoff. Sodium ions were added to neutralize the negative charge of the protein. After the position-restrained molecular dynamics runs were completed, a 10-ns unrestrained molecular dynamics simulation was run, and g_rmsf was used to perform the root mean square fluctuation (r.m.s.f.) calculations. g_covar was performed on all trajectories to generate covariance matrices for backbone atoms only. Principal component analysis (PCA) was performed with g_anaeig to transform the covariance matrices and to generate eigenvalues and eigenvectors for all trajectories. Visual molecular dynamics (VMD) was used to visualize the trajectories (33). PyMOL was used to generate the movies of the trajectories (34).

RESULTS

We previously proposed that the N-terminal neck and C-terminal neck mimic of the non-catalytic Vik1 MHD were part of a communication route that conveyed information about the catalytic state of Kar3 to Vik1 to allow motility of the Kar3Vik1 heterodimer (19). To confirm the relevance of the neck mimic of Vik1 to the motility of Kar3Vik1 motors, a panel of mutants involving residues that follow the conserved C-terminal helix α6 of CgVik1 (Fig. 2A) were made by site-directed mutagenesis and were analyzed for their effects on the mechanochemical properties of recombinant Kar3Vik1 complexes. Two of the mutations targeted Ile578, which forms a hydrophobic interaction with the MHD core of CgVik1 (Fig. 2B). Ile578 was replaced by phenylalanine to ascertain the effect of stabilizing the interaction this residue makes with the core during neck mimic docking, as phenylalanine can form stacking interactions with other buried aromatic side chains. Substitution of Ile578 with alanine was done to learn the effects of destabilizing this interaction. The other mutations targeted Asn580, whose side chain occupies the narrow space between the MHD core and the pivot glycine (Gly322) at the base of the neck (Fig. 2C). This residue is in close proximity to the Glu319—Arg325 salt bridge that helps stabilize neck-core interactions. Substitution of Asn580 with alanine was performed to examine the effects of nullifying its electrostatic interactions with this bond. Conversely, mutations to lysine and aspartic acid were generated to enable interactions of the neck mimic with other residues, which could promote or sterically block neck isomerization. Finally, deletion of all seven residues C-terminal to helix α6 (Δ578–584) was done to establish the overall importance of the neck mimic in motor function. The point mutants and the deletion mutant were each coexpressed and co-purified with a WT CgKar3 construct (CgKar3-CC+N+MD) (Fig. 3A). Denaturing gel electrophoresis of the purified motors resolved two bands of equal intensity and with the expected sizes of Kar3 and Vik1 (Fig. 3B), and CD analysis of these heterodimers showed that all motors exhibited similar folding characteristics, which were indistinguishable from those of WT Kar3Vik1 (Fig. 3C).

Vik1 Neck Mimic Mutations Impede the Ability of Kar3Vik1 to Glide Microtubules—To examine the contribution of the Vik1 neck mimic to Kar3Vik1 motility, microtubule-gliding tests were performed in which motors were affixed to perfu-
sion chamber coverslips via antibodies that recognize the N-terminal polyhistidine tag on the Vik1 subunit. Thereafter, rhodamine-labeled microtubules were bound to immobilized motors in the presence of MgAMPPNP. Following a brief incubation, unbound microtubules were washed from the chamber, and microtubule gliding was initiated by the addition of 1.5 mM MgATP in buffer that either lacked or included additional motor protein. Each of the mutants was tested in parallel with WT Kar3Vik1 under the same conditions (supplemental Movies S1 and S2).

The Δ578–584 deletion mutant showed no microtubule-gliding ability in the absence of additional motor, and we consistently observed many fewer microtubules bound to the coverslip (supplemental Movie S3). When additional motor was provided in the wash buffer used to expel unbound microtubules and initiate gliding, the gliding velocity of this mutant was only 45% of that of the WT (supplemental Movie S4). Likewise, the I578F mutant exhibited minimal motility without additional motor (supplemental Movie S5). However, gliding by I578F was equivalent to that by the WT when washed with a motor-containing buffer (supplemental Movie S6). The other mutant motors showed less severe decreases in microtubule-gliding velocity when additional motor was not provided (~50% of the rate of the WT) (Table 1), but the vast majority of the microtubules were stationary (supplemental Movies S7, S9, S11, and S13). Those that did move either halted intermittently or dissociated after short runs. However, when additional motor protein was included, we observed very few stationary

FIGURE 2. Sequence alignment of Vik1 proteins and design of Vik1 constructs. A, domain architecture of full-length CgVik1 is shown in a bar diagram. Sequence alignment of the CgVik1 neck and neck mimic region with those of representative Vik1 proteins from Ascomycetes yeast was generated by neighbor joining using percent identity in Jalview from ClustalW and is shown above the bar diagram (S4, S5). Gray shading represents the sequence conservation. Ile578 and Asn580 are shown in magenta, along with related residues in other Vik1 proteins. Shown below the bar diagram are sequences of the C termini of the WT and mutant CgVik1 constructs used in this study. Sc, S. cerevisiae; Nc, Neurospora crassa; Kw, Kluyveromyces waltii; Kl, Kluyveromyces lactis; aa, amino acids. B and C show graphic representations of the CgVik1–N–MHD structure (PDB ID 4GKO, chain A) highlighting hydrophobic and polar interactions made by Ile578 and Asn580 with the MHD core and neck-core junction, respectively.
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TABLE 1

| Kinesin construct | No additional motor | Additional motor |
|-------------------|---------------------|-----------------|
|                   | Velocity* | n* | Velocity* | n  |
| Kar3 motor domain | NA       |    | NA       |    |
| WT Kar3Vik1       | 4.37 ± 0.07 | 124 | 4.79 ± 0.02 | 280 |
| Kar3Vik1 I578A    | 2.54 ± 0.13 | 29  | 3.85 ± 0.06 | 390 |
| Kar3Vik1 I578F    | <0.5     | 5  | 4.93 ± 0.02 | 252 |
| Kar3Vik1 N580A    | 2.59 ± 0.12 | 27 | 4.12 ± 0.11 | 167 |
| Kar3Vik1 N580D    | 2.35 ± 0.12 | 36 | 4.97 ± 0.04 | 105 |
| Kar3Vik1 N580K    | 2.71 ± 0.12 | 55 | 4.84 ± 0.03 | 228 |
| Kar3Vik1∆578–584  | No motility |    | 2.15 ± 0.04 | 60  |

* Velocity is reported as the mean ± S.E. and represents assays performed with at least two independent preparations of motor.
* n, number of microtubules tracked; NA, not applicable due to the inability of the construct to be motile.

One explanation for these observations is that most Kar3Vik1 motors with neck mimic mutations are more sensitive to drag forces developed by nonspecific interactions between the microtubule and coverslip. Another is that, in a significant fraction of the motors attached to the coverslip, the Vik1 mutations place the Kar3 subunit in a suboptimal orientation for full activity. A third consideration is that the Vik1 mutations create a defect in the microtubule release mechanism of the Vik1 subunit. For all of these possibilities, we suspect that the second infusion of motors provides an assisting force to start and perpetuate movement because these additional motors are better oriented for microtubule engagement and force production by Kar3 and are less apt to be trapped in a Vik1-tethered state. Pre- and post-powerstroke forms of such a microtubule-bound Kar3Vik1 configuration have recently been characterized by cryo-EM (35, 36).

Interestingly, we also found that even with this apparent "rescue" of normal motility after additional motor infusion, the number of gliding microtubules decreased significantly between the beginning and end of the experiment for many of the mutants (Fig. 4). For the N580D and N580K mutants, about 20%–25% of the microtubules remained in the field of view at the 7.5-min time point. For I578A and N580A, only about 30%–40% of the microtubules were visible by this time. Only about 40% of the microtubules showed continuous gliding for the ∆578–584 deletion mutant, but this number did not drop as appreciably as for the other mutants. This suggests that mutations in the neck mimic impair some aspect of the mechanochemical cycle of Kar3Vik1 that relates to their microtubule interactions or coordination of force production between multiple motors. These results also suggest that the severity of the defect differs depending on the type of mutation. Interestingly, the I578F mutant did not show the same defect in continuous microtubule gliding as the other point mutants when the wash step was supplemented with more I578F mutant protein.

ATP Turnover by Kar3Vik1 Motors Is Dramatically Reduced When the Vik1 Neck Mimic Is Mutated or Deleted—To acquire more specific insights into how the Vik1 neck mimic mutants impair the motility of Kar3Vik1 dimers, their ATPase activities were measured as a function of microtubule and ATP concentration at steady state (Fig. 5). The $k_{cat}$, $K_{0.5}(M_{T})$, and $K_{m}(ATP)$ values for each construct are summarized in Table 2, along with the values for WT Kar3Vik1 and the Kar3 motor domain alone.

microtubules for these mutants (supplemental Movies S8, S10, S12, and S14). Under these conditions, their gliding velocities were also nearly equal to that of WT Kar3Vik1 (Table 1).
The I578A and I578F mutants both exhibited an ~11-fold decrease in $k_{\text{cat}}$ relative to WT Kar3Vik1 and an ~5-fold reduction compared with the Kar3 motor domain. Similarly, the $k_{\text{cat}}$ of N580D was depressed by ~10-fold, whereas N580A and N580K showed more moderate reductions of ~2.9- and ~4.5-fold relative to WT Kar3Vik1, respectively. Deletion of the neck mimic altogether reduced $k_{\text{cat}}$ by 12-fold. Surprisingly, the $K_{\text{m(ATP)}}$ of all mutants was similar to that of WT Kar3Vik1 or even lower in the case of the N580D mutant. This suggests that, in the bulk ATPase assay, the mutant complexes bind microtubules with similar affinity to WT Kar3Vik1 and, thus, their ATPase defect may not be a result of abnormal activation of ATP turnover by microtubules. Instead, the defect may be a consequence of the mutant Vik1 subunit impeding ATP turnover in Kar3. In support of this, all mutants showed unique ATP concentration-dependent activities ($K_{\text{m(ATP)}}$) compared with WT Kar3Vik1. For I578A, I578F, N580A, and N580D and the Δ578–584 deletion mutant, we measured a much lower $K_{\text{m(ATP)}}$ relative to WT Kar3Vik1 (26.5 μM), suggesting that they cause a significant strengthening of ATP binding. Conversely, N580K seems to weaken ATP binding, giving a $K_{\text{m(ATP)}}$ of 42.1 μM.

These results suggest that altering either the hydrophobic interface between the neck mimic and MHD core or the electrostatic interactions between the neck mimic and neck-core junction of Vik1 compromises the catalytic function of Kar3Vik1. Given the remote location of the Vik1 neck mimic mutations from the ATP-binding pocket of Kar3, it is difficult to predict how they could affect ATP binding and slow ATP turnover. It is possible that the ATP-binding site of Kar3 is influenced by altered interactions between peripheral parts of the Kar3 motor domain and the mutant Vik1 subunit. A more likely scenario is that the Vik1 mutants exhibit a microtubule-binding mode or conformational dynamics of their N and C termini that are not conducive to the powerstroke phase of the Kar3 mechanochemical cycle.

**Structural Rearrangements of the Neck and Neck Mimic of Vik1 Are Co-dependent**—Because motions of protein atoms and water molecules and the networks of interactions between them are highly complex, the structural effects of the Vik1 mutations cannot be easily predicted from static crystal structures or measured analytically. Molecular dynamics simulations provide a means to animate molecular models in ways that offer insight into the structural changes that specific functional elements could experience when their interactions are altered. Thus, to better understand the impact of the neck mimic mutations on the dynamic properties of Vik1, we performed molecular dynamics simulations on WT and mutant versions of the CgVik1-N+MHD crystal structure (PDB ID 4GKQ, chain A) (19).

The mutated structures were energy-minimized, and the explicit water molecules, as well as the entire system, were equilibrated prior to 10-ns molecular dynamics simulations at 298 K. Analysis of the WT Vik1 trajectories shows a net downward rotation of the neck, away from the small B1-lobe (supplemental Movie S15). Shortly after this occurs, the C-terminal carboxyl group of Lys$^{384}$ forms transient H-bonding and ionic interactions with Ser$^{316}$ of the neck and Arg$^{388}$ of the core. This extends the antiparallel interaction between the neck and neck mimic beyond that seen in previous Vik1 crystal structures, giving a short-lived intermediate that is reminiscent of the cover-neck bundle in kinesin-1 (6). What stands out in this simulation is that Ile$^{578}$ and Asn$^{580}$ in the neck mimic exhibit nominal changes in their position relative to the starting structure.

**FIGURE 4.** Duration of continuous microtubule gliding by Kar3Vik1 motors with Vik1 neck mimic mutations. The graph shows the percentage of the starting microtubules that exhibited sustained gliding when additional motor was included after Kar3Vik1-microtubule complexes were formed within the gliding assay perfusion chamber. I578A (n = 390) and N580A (n = 167) showed a steady decrease in the number of continuously gliding microtubules, with many microtubules detaching from the coverslip surface or pivoting about a single point. N580D (n = 105) and N580K (n = 228) showed normal microtubule gliding for the first ~5 min, after which microtubules were observed to detach from the coverslip. For I578F (n = 252), ~15% of the microtubules detached very early during the experiment, but the remaining microtubules showed relatively normal continuous movement. For the Δ578–584 deletion mutant (n = 196), only ~40% of the microtubules were motile and showed continuous gliding. $n$ = number of microtubules tracked. Microtubule-gliding velocities are shown in Table 1.
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![Image of a graph showing ATPase rate versus microtubule concentration](image)

**TABLE 2**

| Kinesin construct | Microtubule-activated ATPase kinetics | $k_{cat}$ | $k_{cat}$(SMT) | $K_{m}$ (ATP) |
|-------------------|-------------------------------------|----------|---------------|--------------|
| Kar3 motor domain | WT                                  | 0.39 ± 0.02 | 5.0 ± 0.5     | 11.0 ± 2.0   |
| Kar3Vik1          | WT                                  | 0.95 ± 0.03 | 5.8 ± 0.1     | 26.5 ± 5.3   |
| Kar3Vik1 I578A    | 0.08 ± 0.004                        | 0.53 ± 0.1 | 14.9 ± 1.8    |              |
| Kar3Vik1 I578F    | 0.09 ± 0.005                        | 0.63 ± 0.2 | 4.8 ± 1.6     |              |
| Kar3Vik1 N580A    | 0.21 ± 0.013                        | 0.74 ± 0.2 | 6.6 ± 2.3     |              |
| Kar3Vik1 N580D    | 0.08 ± 0.003                        | 0.25 ± 0.1 | 2.6 ± 1.3     |              |
| Kar3Vik1 N580K    | 0.33 ± 0.016                        | 0.62 ± 0.2 | 42.1 ± 8.8    |              |
| Kar3Vik1Δ578-584  | 0.08 ± 0.003                        | 0.41 ± 0.1 | 6.3 ± 4.4     |              |

Principal component analysis was used to analyze the overall atomic fluctuations of all trajectories. Transformation of the covariance matrix results in eigenvalues and a corresponding set of eigenvectors. Using the first eigenvector, which contains the most atomic fluctuations, extreme structures of the trajectory were generated. The magnitude and direction of the collective motion along the first eigenvector can then be visualized by the direction and length of arrows drawn from the average structure of the trajectory to the extreme structures using PyMOL. Fig. 6 shows that mutations in the neck mimic elicit prominent differences in the collective atomic fluctuations of the neck and neck mimic relative to the WT. This implies that Ile578 and Asn580 are important determinants of neck mimic docking and that movements of the neck are interconnected with those of the neck mimic.

As the eigenvalues correspond only to a fraction of the r.m.s.f. values of the system, the r.m.s.f. for the whole system was calculated independently. Fig. 7A shows an overlay of these r.m.s.f. values for all α-carbons of the mutant and WT forms of the Vik1 MHD. The largest movements are found in the neck and C terminus of Vik1 and in loop regions between strands β4 and β6 and between helices α4 and α5. By subtracting the r.m.s.f. values of each α-carbon of the WT simulation from those of the mutants, we observe significantly increased motion of the neck and neck mimic for I578A and N580D (Fig. 7, B and E). Conversely, I578F exhibits a dramatic decrease in mobility of these elements (Fig. 7C). The other point mutants show more modest changes (Fig. 7, D and F). The main themes arising from these analyses are that increases or decreases in neck and neck mimic motions coincide with one another and that the direction of neck motion is differentially affected by each mutation in the neck mimic of Vik1. As many of the flexible regions in the core were built separately by the comparative protein structure modeling program Modeller (28), confidence in the significance of mutant-specific fluctuations here is naturally lower.

In the molecular dynamics simulation for I578A (supplemental Movie S16), downward rotation of the neck is much more rapid and exaggerated compared with the WT Vik1 trajectory (supplemental Movie S15). The shorter side chain of alanine seems to allow the neck and neck mimic to undock from the MHD core by destabilizing the hydrophobic interaction formed between Ala324 of the neck-core junction and Thr530, Phe548, Phe555, and Leu577 of the core. As this happens, the neck mimic extends, allowing Arg312 to form an electrostatic bond with the terminal carboxyl of Lys584. This produces an antiparallel interaction between the neck and neck mimic that is more long-lived than that seen in the WT trajectory.

The I578F mutant shows the opposite effect of I578A (Fig. 6, B and C) and (supplemental Movie S17). Phe578 immediately forms a π-stacking interaction with Phe548 and a hydrophobic interaction with His549 (data not shown) from helix α5. This causes α6 to migrate away from the β1-lobe and change its alignment against the motor core. This also pulls the C terminus of α5 closer to the motor core. Together, these interactions brace the neck mimic against the neck-core junction and reduce the motion of the neck and terminus of the neck mimic. We speculate that these changes may be the cause of the strong motility defect of the I578F mutant in the microtubule-gliding assay. Rather than weakening the neck-neck mimic interaction as seen in the I578A mutant, this mutant may overly stabilize it...
and thereby create a larger kinetic barrier for Kar3 to overcome to free Vik1 from the microtubule.

The Asn$^{580}$ mutant trajectories were equally informative in modeling potential structural transitions of the elements linking Vik1 to Kar3. For N580A, it was clear that the neck becomes much more mobile in the absence of the polar interactions provided by the asparagine (supplemental Movie S18). Without H-bonding between the side chain of Asn$^{580}$ and the main chain carbonyl of Glu$^{319}$, the Glu$^{319}$–Arg$^{325}$ salt bridge immediately changes conformation, and the downward angle of the neck is no longer maintained (Fig. 6D). Instead, rotation about Gly$^{322}$ places the neck in a conformation similar to molecule B of the CgVik1-N+MHD crystal structure (PDB 4GKQ, chain B) (Fig. 1C).

During the N580D simulation, electrostatic repulsion between the side chain of Asp$^{580}$ and Glu$^{319}$ causes the neck to be displaced from the neck mimic (supplemental Movie S19). Similar to the N580A mutant, there is a lack of H-bonding between Asp$^{580}$ and Glu$^{319}$, which allows for additional conformational freedom of the neck-core junction (Fig. 7E). As a result, a new salt bridge between Glu$^{319}$ and Lys$^{522}$ forms in the simulation and causes the neck helix to pivot upward and to bend toward the MHD core (Fig. 6E). As for N580K, the extended lysine side chain initially positions itself into the small space between the

**FIGURE 6. Visual representation of the results from PCA of molecular dynamics simulations.** The average structures of the WT and all mutant forms of the CgVik1-N+MHD model (PDB ID 4GKQ, chain A) after PCA are shown in ribbon representation with rainbow coloring. The first eigenvector produced by PCA contains the largest conformational changes (variance). The two extreme structures that project along this vector were generated for the WT and mutant forms of Vik1. The arrows represent directional changes between the average structure and the two extreme structures. C-ter, C terminus; N-ter, N terminus.
neck-core junction turn and the Glu$^{319}$–Arg$^{325}$ salt bridge (supplemental Movie S20). At ~2.5 ns during the dynamics simulation, the Lys$^{580}$ side chain is expelled from this small space as the neck and neck mimic separate, and the neck helix rotates downwards (Fig. 6F). Following this, the terminal amino group of Lys$^{580}$ establishes polar interactions with the backbone carbonyls of Glu$^{319}$ and Cys$^{323}$, which seem to help rigidify an antiparallel arrangement of the neck and neck mimic.

These simulations indicate that the neck and neck mimic are dynamic in nature and that their stability is highly co-dependent. The $\Delta$$t$785–$t$844 trajectory best illustrates this (supplemental Movie S21). In the absence of the neck mimic, the remainder of the C terminus (helix $\alpha$6) shows an enhancement of movement relative to the WT, whereas fluctuations in the neck decrease relative to the WT (Fig. 7G).

**DISCUSSION**

Structural and kinetic analyses of Kar3Vik1 and Kar3Cik1 complexes have helped generate models describing the motile mechanism of these heterodimeric motors (19, 35–39). These depict the Vik1 (or Cik1) subunit making the first contact with the microtubule while Kar3 is in an ADP-bound state. Subsequent binding of Kar3 to the microtubule could occur via a change in orientation of the Vik1 $\alpha$-helical neck, at which point, internal strain would presumably develop between Kar3 and Vik1 in their neck elements. This may enable a gating scenario akin to processive kinesins in which Vik1 may limit ADP release, ATP binding, or hydrolysis in Kar3 until Kar3 attains a tight microtubule-binding state (14, 40). Likewise, gating of Vik1 by Kar3 may regulate the timing of the release of Vik1 from the microtubule until the complex is appropriately primed for the powerstroke event (16, 41). At this point, rotation of the coiled coil formed between Kar3 and Vik1 is triggered by exchange of ADP for ATP in Kar3 (35–37). Dissociation of the Kar3Vik1-microtubule complex occurs after ATP is hydrolyzed (35–37).

The striking resemblance between the conformations of the CgVik1 neck and neck mimic to those of bona fide kinesin motor domain suggests that these modules are somehow involved in the Kar3Vik1 mechanochemical cycle (Fig. 1) (3, 4, 19, 42). Because neck mimic docking and undocking in Ncd and KCBP correlate with the high microtubule affinity ATP-bound state and low microtubule affinity ADP-bound state, respectively, we recently proposed that the neck mimic of Vik1 was also a key determinant of Vik1 microtubule affinity (9, 10, 19). However, the data presented here suggest that the Vik1 neck mimic is intimately connected to ATP turnover and force production by Kar3. Alterations to two of the residues involved in annealing the Vik1 C terminus to the MHD core (Ile$^{578}$ and Asn$^{580}$) impaired motility and ATP consumption by Kar3Vik1 but did not appear to weaken microtubule interactions.

Ile$^{578}$ is conserved among Vik1 proteins and has counterparts in most members of the kinesin-14 family (Lee$^{639}$ in *S. cerevisiae* Vik1) (Fig. 2A). With the neck mimic docked, Ile$^{578}$ fits into a hydrophobic cavity formed by the ends of helices $\alpha$4 and $\alpha$5, the core $\beta$-sheet, and the neck-core junction (Fig. 2B). Here, it is positioned similarly to Ile$^{1210}$ in the neck mimic of KCBP and Ile$^{325}$ in the neck linker of kinesin-1 (10, 43). The side chain of Met$^{672}$ at the end of $\alpha$6 in Ncd is similarly positioned in a small hydrophobic cavity against the motor core (9). Based on this conservation, it is somewhat surprising to us that mutation of Met$^{672}$ to asparagine in Ncd did not appreciably hinder its steady-state parameters or microtubule-gliding ability (11). We would expect that conversion to a polar residue would be more severe than changing the size of the hydrophobic side chain, as was our approach for Ile$^{578}$ in Vik1. Deletion of Met$^{672}$ in an Ncd truncation mutant did, however, create a dramatic reduction in microtubule binding, gliding, and ATPase activity, as did replacement of the short stretch of basic residues immediately downstream of Cys$^{670}$ with uncharged residues. This indicates the strict importance of residues adjacent to helix $\alpha$6 in helping zipper the neck mimic to the motor domain core. In this case, electrostatic interactions appear to play a larger role in Ncd neck mimic docking, whereas the hydrophobic Met$^{672}$ may play an alignment role in the C terminus rather than a major adhesion function. Nonetheless, the Vik1 I578A and I578F mutants clearly affected Kar3Vik1 activity, indicating that Ile$^{578}$ makes critical adhesive hydrophobic interactions with the core. Molecular dynamics simulations of the CgVik1-N+MHD crystal structure with a phenylalanine substitution at Ile$^{578}$ support this by the striking enhancement of neck mimic annealing relative to the WT and I578A mutant. Like Ncd, Vik1 proteins also have several basic residues in their C terminus (Fig. 2A), but their importance in Kar3Vik1 function awaits investigation.

At Asn$^{580}$, whose side chain projects toward key electrostatic interactions at the neck-core junction, all amino acid substitutions led to defects in ATP turnover by Kar3Vik1. The N580K mutant had the mildest effect, whereas the N580D substitution was most severe. These results indicate that both the polarity and size of this residue are important for proper functioning of Vik1 during Kar3Vik1 catalysis. Interestingly, the residue equivalent to Asn$^{580}$ in *S. cerevisiae* Vik1 is Lys$^{641}$, and it is followed by a stretch of four other basic residues (Fig. 2A). Thus, it stands to reason that the functional role of Asn$^{580}$ may be partially sustained by a lysine substitution. This may explain why the ATPase activity of the N580K mutation was least affected.

The molecular dynamics simulations of these mutants reveal a possible mechanism for the influence of Asn$^{580}$ on the neck orientation of Vik1, which could impact on Kar3-Vik1 interactions and/or Kar3-microtubule interactions related to motility.

**FIGURE 7.** r.m.s.f. calculations for all molecular dynamics trajectories. A, overlay of the WT (black), I578A (red), I578F (dark blue), N580A (yellow), N580D (magenta), N580K (cyan), and A578–S584 (light green) r.m.s.f. calculations for all C termini, including loops and side chains filled in by Modeller (modeled residues include Gly$^{369}$, Phe$^{371}$, Pro$^{376}$, Asn$^{378}$–Asn$^{385}$, Ile$^{391}$–Glu$^{405}$, Ile$^{407}$–Gln$^{409}$, Thr$^{412}$, Asn$^{413}$–Gly$^{417}$, and Lys$^{439}$). The x axis represents residue number, and the y axis represents r.m.s.f. in Å. Secondary structure elements are represented just below the x axis. B–G, bar graph representations of the r.m.s.f. differences between WT and mutant trajectories by subtracting r.m.s.f. values of the WT from that of the mutants. The x axis represents residue number, and the y axis represents r.m.s.f. differences in Å. The sections comprising the neck and C terminus (C-ter) of each construct are labeled.
Importance of the C Terminus of Vik1

Contrary to the previously proposed role of Asn\textsuperscript{580} in facilitating neck rotation by destabilizing either the Glu\textsuperscript{319}–Arg\textsuperscript{325} or Glu\textsuperscript{319}–Lys\textsuperscript{552} salt bridge (19), the polar interactions it forms at the neck-core junction seem to secure the neck near the core and in a downward orientation. According to the N580A and N580D mutant trajectories, disruption of these polar interactions leads to rapid repositioning of the side chain of Arg\textsuperscript{325} and dramatic changes in the degree and direction of neck movement. Likewise, mutations that alter adhesion of the neck mimic to the MHD core also lead to changes in neck mobility.

On the basis of these results, we propose that neck mimic dynamics of Vik1 are intimately connected to the lever-like rotation of the coiled-coil-forming region of Kar3 that elicits the force-producing powerstroke in Kar3Vik1 (36). Because isomerization of the Vik1 neck has been proposed to encourage Kar3-microtubule interactions (19), changes in the dynamic properties of the Vik1 neck as a result of the nearby neck mimic mutations could be impeding the ability of Kar3 to orient properly on the adjacent α/β-tubulin subunit (35). This may stabilize states of Kar3Vik1 prior to formation of stable Kar3-microtubule interactions, providing a rationale for perturbation of ATPase activity and premature detachment of microtubules by several of the mutants during microtubule-gliding analyses.

An alternate possibility is that the Vik1 mutants may inhibit the powerstroke of Kar3Vik1. Perhaps disruption of normal neck-neck mimic docking and undocking dynamics creates a deficiency in the interhead tension between Kar3 and Vik1 that prolongs the state where both heads are bound to the microtubule: before ADP is exchanged for ATP by Kar3. Slower ADP release would imply such a defect and would support the existence of inhibitory gating of Kar3 (13, 14, 44). Unfortunately, our steady-state kinetic analyses do not provide the temporal resolution required to identify specific steps in the ATPase cycle that are altered by the mutations, and thus, transient state kinetic tests of the mutants will need to be pursued to address this further.

Given that our molecular dynamics simulations were performed with a monomeric Vik1 MHD and not the Kar3Vik1 dimer, it is difficult to conclude with certainty how the neck mimic mutations would impose effects on the Vik1 neck helix when it is attached to the Kar3 neck as a coiled coil. Nonetheless, the simulation studies did show that the neck mimic of Vik1 is highly dynamic in nature and that the interactions it forms with the neck-core junction and MHD core are highly specific. It is not unreasonable then to suggest that, as Kar3 completes its powerstroke, intramolecular strain in the Vik1 neck changes its position relative to the core and in turn profoundly affects the structure of the neck mimic (14, 45–47). This would support the concept of Kar3 having a stimulatory gating effect on Vik1 through strain-induced disintegration of the docked neck-neck mimic motif and dissociation of the Vik1-microtubule interaction (16, 46). By specifically labeling the neck, neck mimic, and MHD core, it may be possible to use fluorescence resonance energy transfer studies to confirm and pinpoint the timing of Vik1 neck rotation and neck mimic docking/undocking events during the Kar3Vik1 mechano-chemical cycle (3, 48, 49).

We do not fully understand how influx of additional motors in the final step of the microtubule-gliding assay gives near-normal gliding properties to most of the mutants. Although we lack the ability to visualize the motors beneath the microtubules, we propose that the secondarily added motors can attain conformations that are able to circumvent the defects imposed by the mutant Vik1 subunit. This assumes that the presence of adherent microtubules favors binding of the secondarily added Kar3Vik1 motors via the Kar3 subunit only, or via a more canonical orientation of the Kar3 and Vik1 heads. In this way, some of these motors could bypass initial motion-impeding Vik1-binding events and rescue motility defects created by the Vik1 mutants. Precedent for complementation of an inactivate subunit with a functional subunit in motility assays has been demonstrated previously for kinesin-1 (16, 50). However, the applicability of these findings to our investigations with Kar3Vik1 mutants is limited by the processive nature of kinesin-1 and by the fact that both kinesin-1 subunits used to create the motors in these studies had the capacity to bind nucleotide. We also point out that microtubule gliding by some of our Kar3Vik1 mutants exhibited similarities to an S. cerevisiae Kar3Vik1 construct that was designed to restrict separation of the heads by including a cross-link at the bottom of the neck coiled coil (35). These microtubules detached prematurely from the coverslip, presumably due to an inability of the Kar3 and Vik1 subunits to engage them simultaneously (35).

Our observation that the microtubule-stimulated ATPase activity of the Kar3Vik1 mutants is low compared with their gliding velocity upon extra motor addition may be attributable to the fundamental differences between the ATPase and microtubule-gliding assays. In the gliding assays, kinesins are fixed to a specific surface with their motor domains projecting toward the space filled with microtubules. Here, individual motors may cooperate to move microtubules, particularly if multiple Kar3 heads form a stable microtubule interaction and coordinate powerstroke events as an ensemble. In the ATPase assay, however, each Kar3Vik1 dimer can be assumed to function independently of the other molecules. The occurrence of ATP turnover events relies on the ability of each Kar3 subunit to form a stable and catalytically competent interaction with the microtubule in solution, without assisting forces or proximity effects of nearby motor ensembles. In line with our observations, a mutation in the neck of the neuronal kinesin-1 KIF5A (A361V) showed no deficits in the microtubule-gliding properties of the motor but reduced the steady-state ATPase \( k_{\text{cat}} \) to half that of the WT (51). Similarly, disruption of the Glu\textsuperscript{319}–Arg\textsuperscript{325} and Glu\textsuperscript{319}–Lys\textsuperscript{552} salt bridges between the neck and core of CgVik1 via the E319A mutant also produced Kar3Vik1 motors with near-normal gliding function but almost 4-fold lower ATPase activity compared with WT Kar3Vik1 (19).

In summary, our studies reaffirm the dependence of Kar3 on Vik1 for full activity and provide new insights into the structural elements in Vik1 that pertain to Kar3Vik1 mechanochemistry. Our results also support the idea that structural elements distant from one another in dimerized motor domains are tightly integrated such that one subunit affects the catalytic performance of the partner subunit. Although Vik1 cannot
bind ATP, it utilizes the same canonical elements found at the N and C termini of all kinesins for motor function. How interaction of these elements germinated into the force-producing domain of kinesins is unclear, but it is interesting that early Vik1 proteins retained this structure in favor of a pocket for ATP. Additional mechanistic studies of mutant versions of Vik1 and Kar3 will be needed to further evaluate the structural and kinetic relationships underlying involvement of their N and C termini for precise coordination of Kar3Vik1 movement.

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