Long-range electrostatic interactions significantly modulate the affinity of dynein for microtubules

Ashok Pabbathi,1 Lawrence Coleman,1 Subash Godar,1 Apurba Paul,1,2 Aman Garlapati,3 Matheu Spencer,1 Jared Eller,1,4 and Joshua Daniel Alper1,2,5,*

1Department of Physics and Astronomy, Clemson University, Clemson, South Carolina; 2Eukaryotic Pathogen Innovations Center, Clemson, University, Clemson, South Carolina; 3School of Mathematical and Statistical Sciences, Clemson University, Clemson, South Carolina; 4Department of Genetics and Biochemistry, Clemson University, Clemson, South Carolina; and 5Department of Biological Sciences, Clemson University, Clemson, South Carolina

ABSTRACT The dynein family of microtubule minus-end-directed motor proteins drives diverse functions in eukaryotic cells, including cell division, intracellular transport, and flagellar beating. Motor protein processivity, which characterizes how far a motor walks before detaching from its filament, depends on the interaction between its microtubule-binding domain (MTBD) and the microtubule. Dynein’s MTBD switches between high- and low-binding affinity states as it steps. Significant structural and functional data show that specific salt bridges within the MTBD and between the MTBD and the microtubule govern these affinity state shifts. However, recent computational work suggests that nonspecific, long-range electrostatic interactions between the MTBD and the microtubule may also play an important role in the processivity of dynein. To investigate this hypothesis, we mutated negatively charged amino acids remote from the dynein MTBD-microtubule-binding interface to neutral residues and measured the binding affinity using microscale thermophoresis and optical tweezers. We found a significant increase in the binding affinity of the mutated MTBDs for microtubules. Furthermore, we found that charge screening by free ions in solution differentially affected the binding and unbinding rates of MTBDs to microtubules. Together, these results demonstrate a significant role for long-range electrostatic interactions in regulating dynein-microtubule affinity. Moreover, these results provide insight into the principles that potentially underlie the biophysical differences between molecular motors with various processivities and protein-protein interactions more generally.

INTRODUCTION

Cytoplasmic dynein (hereafter called dynein unless otherwise specified) is a microtubule minus-end-directed motor protein that drives diverse functions in eukaryotic cells, including retrograde intracellular transport (1), mitotic spindle assembly (2), chromosome segregation (3), nucleus positioning (4,5), and cytoskeletal network organization (6). Dynein dysfunction is associated with several diseases; in particular, neurodegenerative diseases, including Alzheimer’s, Parkinson’s, and Huntington’s disease (7–15). Cytoplasmic dynein is a 1.4 MDa protein complex with two copies of a 530 kDa heavy chain and associated regulatory light, intermediate light, and intermediate chains (16).
The dynein heavy chain consists of a dimerizing tail domain, a linker, and a microtubule-binding domain (MTBD) separated from a ring of six AAA⁺ domains by a 15 nm coiled-coil stalk (Fig. 1 A) (16,17). Communication between the ATPase active site in AAA1 and MTBD through the coiled-coil stalk modulates the binding affinity of dynein toward microtubules (18,19).

Processivity, which is a measure of how far a motor protein moves along its associated filament before detaching (20), is an essential biophysical property affecting motor proteins’ cellular functionality. Dynein processivity depends on the details of the affinity of the MTBD for the microtubule (21); it must switch between high- and low-binding affinity states as a function of ATP hydrolysis state in a coordinated way to walk along a microtubule (21,22). The affinity of dynein for microtubules switches between high- and low-binding affinity states due to conformational changes in the MTBD, which results from changing registrations of the coiled-coil domain helices (19,22–25) in response to the ATPase cycle state in AAA1 (19,22,26,27). It is thought that dynein switches to its high-binding affinity state when it is unbound from the microtubule, causing it to bind, and that it switches to its low-binding affinity state when it is bound to the microtubule, causing it to unbind and step forward (28,29).

Electrostatic interactions dominate the dynein-microtubule binding affinity mechanisms. Charged-to-neutral and charge-flipping amino acid substitutions in the MTBD’s microtubule-binding interface alter the microtubule-binding affinity (22,28,30), presumably due to changes in surface charge complementarity (31,32). In addition, multiple dynamic salt bridges within the MTBD and between the MTBD and the microtubule regulate the MTBD-microtubule-binding affinity (24). Together, these results indicate the importance of both specific and nonspecific electrostatic interactions in the biophysical mechanisms of dynein.

Previously, our computational work on the dynein MTBD-microtubule-binding interface suggested that nonspecific, long-range electrostatic interactions (which occur when the distance between interacting partners is greater than a few Angstroms and thus longer than salt bridges or hydrogen bonds) contribute significantly to the MTBD-microtubule-binding affinity (33). When we computationally mutated multiple charged amino acids that are remote from the binding interface to neutral ones, we found that the changes in the electrostatic component of binding energies are similar in magnitude to changes associated with mutations to the previously identified specific dynamic salt bridges (33). Moreover, our computational investigation of interactions between tubulin’s highly charged C-terminal tails (or E-hooks) and the MTBD further indicated the importance of long-range, nonspecific electrostatic interactions in guiding dynein-microtubule binding (34). Therefore, we hypothesize that long-range electrostatic interactions, i.e., those remote from the binding interface, contribute significantly to the MTBD-microtubule-binding affinity and thus play an important role in the motility mechanisms of dynein.

To investigate the effect of long-range electrostatic interactions on the binding of dynein to microtubules, we characterized the binding affinity of wild-type (WT) and long-range electrostatic interaction-altering mutant MTBDs (Fig. 1 B) for microtubules in the high-binding affinity (22) state based on our computational work (33). We measured the equilibrium binding affinity using microscale thermophoresis (MST) and the force-dependent unbinding rate using optical tweezers in varying concentrations of added salt. We found that mutating a negatively charged amino acid remote from the binding interface to a neutral one enhanced the binding
affinity and reduced the force-dependent unbinding rate of the MTBD in an ionic strength-dependent manner. Together, our results suggest a role for long-range electrostatic interactions in regulating dynein’s processivity.

MATERIALS AND METHODS

Tubulin purification and labeling

Tubulin was purified from porcine brains using a phosphocellulose column (PC-tubulin, a gift from Jonathon Howard, Yale University) (35). We cycled (polymerized and depolymerized) and rhodamine labeled the PC-tubulin using standard protocols (36). In brief, we incubated a 10-fold excess of rhodamine dye (56)-TAMRA, SE, Biotium, Fremont, CA) with a microtubule solution in HEPES labeling buffer (0.1 M NaHEPES [pH 8.6], 4 mM MgCl₂, 1 mM EGTA, 40% [v/v] glycerol) for 40 min at 37°C. We then depolymerized the labeled microtubules at 4°C and pelleted any remaining microtubules. We cycled the labeled tubulin again to ensure that all the labeled tubulin was polymerization competent. We measured the degree of labeling from the concentration of tubulin and the TAMRA dye using a UV-vis spectrophotometer (NanoDrop One, Invitrogen, Waltham, MA) with an extinction coefficient for tubulin at 280 nm of 115,000 M⁻¹ cm⁻¹, a correction factor of 0.3, and an extinction coefficient for the TAMRA dye at 555 nm of 65,000 M⁻¹ cm⁻¹. We found that the degree of labeling was 0.8 rhodamine molecules per tubulin dimer.

Microtubule preparation

We prepared taxol-stabilized, unlabeled microtubules using standard methods, with slight modifications (37). In brief, we added unlabeled tubulin (32 μM) to polymerization buffer (4% DMSO, 4 mM MgCl₂, 1 mM GTP, and BRB80 [80 mM PIPES, 1 mM EGTA, 1 mM MgCl₂ (pH 6.9)]) for 30 min at 37°C to polymerize microtubules. We diluted the microtubules in BRB80T (BRB80 with 20 mM MgCl₂, 1 mM EGTA, 40% [v/v] glycerol) for 40 min at 37°C to 1 cm³, a correction factor of 0.3, and an extension coefficient for the TAMRA dye at 555 nm of 65,000 M⁻¹ cm⁻¹. We found that the degree of labeling was 0.8 rhodamine molecules per tubulin dimer.

MTBD expression and purification

The SRS-MTBD (22/19), AB (monomer), WT plasmid, which encodes a chimeric seryl-tRNA synthetase (SRS)—mouse cytoplasmic dynein MTBD expression and purification

We expressed the MTBD constructs in E. coli by overnight induction with 0.5 mM IPTG at 18°C. We harvested the cells by centrifugation (4000 × g for 25 min), resuspended them in binding buffer (20 mM sodium phosphate, 500 mM NaCl, and 40 mM imidazole [pH 7.4]), and lysed them using an ultrasonic tip sonicator. We clarified the lysate by centrifugation (4000 × g for 35 min) and purified the MTBD constructs from the lysates using a HisTrap High Performance Nickel Sepharose column (17-5247-01, Cytiva [formerly GE Healthcare Life Sciences], Marlborough, MA), as per the manufacturer’s instructions. In brief, we loaded the sample onto the column, washed it with 5 column volumes, and eluted the 6× His-tagged MTBDs with an elution buffer (20 mM sodium phosphate, 500 mM NaCl, and 500 mM imidazole [pH 7.4]) in 1 column volume fractions. We pooled fractions containing the purified protein, desalted the pooled fractions using PD-10 desalting columns packed with Sephadex G-25 resin (17085101, Cytiva [formerly GE Healthcare Life Sciences]), and stored aliquots in PBS with 10% glycerol at −80°C. Densitometry of Coomassie blue-stained SDS-PAGE gel electrophoresis showed approximately 90% purity of each MTBD construct used (Fig. S1 in the supporting material).

To test the long-range electrostatic interaction hypothesis, the charge-altering mutations remote from the binding interface must not change other significant contributing factors to binding affinity, like the structure. We assessed the structural stability of the MTBD constructs to mutations in these charged residues using circular dichroism (Fig. S2 in the supporting material) and computational methods (Dynafun) (39) and Site-Directed Mutator (SDM) (40). We found that the D3402A mutation did not destabilize the MTBD but that the E3320A mutation caused significant structural changes (see supporting material for details). Therefore, we proceeded to make binding affinity and dissociation measurements with the D3402A construct only.

MST binding affinity measurements

MST measures the dissociation constant (K_D) of a biomolecular interaction based on changes in thermophoretic mobility, which is the directed movement of molecules in a microscopically temperature gradient when one fluorescently labeled molecule binds to an unlabeled binding partner (41). We used MST because it requires significantly less sample than alternative techniques such as isothermal calorimetry (41). We measured the binding affinity of MTBDs (the fluorescently labeled target) to taxol-stabilized, unlabeled microtubules (the unlabeled ligand) using a Monolith NT.115 MST system (NanoTemper Technologies, Munich, Germany). We labeled the MTBDs with a 2nd-generation RED-trisNTA His Tag Labeling Kit (MO-L018, NanoTemper Technologies) as per the manufacturer’s instructions with slight modifications (42). In brief, we mixed equal volumes of purified MTBD protein (200 nM) and RED-trisNTA dye (100 nM) and incubated them overnight at 4°C. Due to the excess of MTBD and the high affinity of the dye for the MTBD’s His Tag, these labeling conditions ensured that the dye molecules bound to the MTBDs in 1:1 stoichiometry (42). Then, we removed protein and dye aggregates from the sample by centrifugation (18,000 × g) at 4°C for centrification (4000 × g for 25 min), resuspended them in binding buffer (20 mM sodium phosphate, 500 mM NaCl, and 40 mM imidazole [pH 7.4]), and lysed them using an ultrasonic tip sonicator. We clarified the lysate by centrifugation (4000 × g for 35 min) and purified the MTBD constructs from the lysates using a HisTrap High Performance Nickel Sepharose column (17-5247-01, Cytiva [formerly GE Healthcare Life Sciences]), and stored aliquots in PBS with 10% glycerol at −80°C. Densitometry of Coomassie blue-stained SDS-PAGE gel electrophoresis showed approximately 90% purity of each MTBD construct used (Fig. S1 in the supporting material).
10 min. We diluted the supernatant, which consisted of labeled MTBD, to 50 nM and proceeded with MST.

To perform MST, we prepared a 16-step twofold serial dilution series of the taxol-stabilized, unlabeled microtubules into BRB80T. The series started with the equivalent of approximately 23 nM tubulin dimers and ended with approximately 0.6 nM tubulin dimers in solution, but with those dimers polymerized in the microtubules. We incubated each sample from the serial dilution, as well as a no microtubule control, with 50 nM of RED-tris-NTA-labeled MTBD in BRB80T with 0.2% IGEPAL CA-630 (J61055, Alfa Aesar). To further probe the effect of long-range electrostatic interactions on the $K_d$, we performed MST in samples with 0, 100, and 300 mM of KCl added. These salt concentrations decrease the Debye length, i.e., the characteristic length scale by which the electrostatic effects decay in the solvent, from 0.8 to 0.6 nm and 0.4 nm, respectively, per

$$\lambda_D = \sqrt{\frac{\varepsilon k_BT}{\sum_i c_i q_i^2}},$$  (1)

where $\varepsilon$ is the dielectric constant of the solvent (80.4 for water), $k_BT$ is the scale factor for molecular energy (Boltzmann's constant times absolute temperature $= 4.11 \times 10^{-21}$ J), $c_i$ is the concentration of charged species, $q_i$ is the charge carried by species, $i$, and $N$ is the total number of mobile charged species in solution. However, the additional KCl is not a sufficient cause to quench the Hofmeister (43) and Kirkwood 'salting out' effect (44,45).

We transferred the samples to glass capillaries tubes (MO-K022, NanoTemper Technologies). We acquired MST traces, which track fluorescence intensity changes due to thermophoresis in a confocal laser-induced temperature gradient within the capillaries (41), at 40% excitation power of Nano-RED LED. Using MO.Affinity Analysis (NanoTemper Technologies), we fit the traces to a linearized model of MST and found the fraction of bound MTBD. We fit the data to the Hill-Langmuir equation (46) to find $K_d$ of the fluorescently labeled target MTBDs for the unlabeled ligand microtubules. In doing so, we excluded MST traces that showed evidence of problems with the MST data (e.g., aggregation) as flagged by the MO.Control software (NanoTemper Technologies), optimized the “On Time” for each fit by maximizing the signal-to-noise ratio, and used the concentration of MTBD (50 nM) as a fixed parameter. We performed each MST two to four times for each condition and calculated the mean $K_d \pm$ the standard error, propagating the standard error of the fit through the mean and accounting fitting to log-linear data.

Conjugation of MTBD to beads for force-dependent dissociation optical tweezers assays

We used a biotinylated anti-His Tag antibody-streptavidin method to conjugate the MTBDs to polystyrene beads for the force-dependent dissociation optical tweezers assays (47). In brief, we washed 1% (w/v) 1.04 μm diameter, streptavidin-coated polystyrene microspheres (CP01004, Bangs Laboratories, Fishers, IN) three times with centrifugation (11,000 × g, 3 min) and resuspension in PBS with 0.1% Tween 20 (V0777, AMRESCO LLC., Solon, OH). We incubated the washed beads with 0.25 mg/mL mouse monoclonal biotinylated anti-His Tag monoclonal antibody (AS-61250-LLC., Solon, OH). We incubated the washed beads with 0.25 mg/mL mouse monoclonal biotinylated anti-His Tag monoclonal antibody-streptavidin method to conjugate the MTBDs to polystyrene beads for the force-dependent dissociation optical tweezers assays. We used power spectral density analysis on the position of the trapped bead to calibrate the trap stiffness for each bead (0.09–0.1 pN/nm) (50). In brief, we converted the QPD voltage into the displacement of the bead from the trap center using quadrant photodiode-based (QPD, QP45-Q-HVSD, First Sensor, AG, Berlin, Germany) back focal plane detection (49).

Force-dependent dissociation assays

We prepared flow cells with channels using pinaraha-cleansed silanized coverslips (22 × 22 mm), as described previously (51). We washed the flow channel with BRB80 and then incubated 0.2 mg/mL anti-rodamin (TRITC) antibody (A-6397, Invitrogen) in it for 5 min. We washed out excess antibody with BRB80 and then incubated the flow channel with 1% Pluronic F-127 (PK-CA707-59000, PromoCell, Heidelberg, Germany) for 5 min to passivate the surface. We washed out excess Pluronic F-127 with BRB80 and then incubated the flow channel with a polarity-marked microtubule solution diluted in BRB80T for 5 min. We washed out excess microtubules with BRB80T. We 20-fold diluted the MTBD-coated beads in Tris assay buffer with anti-fad agents (125 nM glucose oxidase [G2133, Sigma-Aldrich, St. Louis, MO], 32 mM catalase [C9322, Sigma-Aldrich], 40 mM D-glucose [VWR10188, VWR Life Science, Solon, OH], 1% β-mercaptoethanol [VWRVM131, VWR Life Science]) and flowed them into the channel. We sealed the flow channel with nail polish to prevent the evaporation of the solvent.

The optical tweezers and its calibration

We performed force-dependent binding affinity assays using an optical tweezers with a 1064 nm, 10 W ytterbium fiber laser (YLR-10-1064-LP, IPG Photonics, Oxford, MA) focused on the sample plane by an oil immersion objective (CFH60 Plan Apochromat Lambda 60× N.A. 1.4, Nikon Instruments, Melville, NY), as described previously (48). Multiple polarizing beam splitters, zero-order half-wave plates, beam traps, and neutral optical density filters (ThorLabs, Newton, NJ) direct, condition, and regulate the laser power at the sample plane. We monitored the power of the trapping laser at the sample plane by measuring the power of the laser before entering the objective with a photodiode sensor (S121C, Thorlabs), which we correlated to the power at the sample plane using a microscope slide thermal power sensor (S276C, Thorlabs) and power meter interface (PM100USB, Thorlabs). We used 20–25 mW laser power (as measured at the sample plane) in our experiments.

An integrated micropositioner/nanopositioner (MicroStage and Nano-LP100, Mad City Labs, Madison, WI) driven by USB3 controllers (Micro-Drive2 and Nano-Drive3, respectively, Mad City Labs) positions and focuses the sample on the image plane of the objective. A Grasshopper3 1.5 MP Mono USB3 Vision CCD camera (imaging chip: ICX825, Sony Semiconductor Solutions Corporation, Atsugi, Japan); camera: GS-U3-1555M-C, FLIR Systems, Wilsonville, OR) monitors the sample under test. Bright-field Köhler transmission illumination generated by a LuxeonStar 1030 mW Royal-Blue (448 nm) Rebel LED on a SinkPAD-II 20 mm Star Base (SP-01-V4, Quadica Developments, Lethbridge, Canada) through a series of optical (lenses and irises) and supporting mechanical condenser elements (Thorlabs) and a high N.A. oil condenser (TF-HNA-OIL, High N.A. DIC Lens, Oil N.A. 1.40, Nikon Instruments, Melville, NY) illuminates the beads, and fluorescence excitation generated by an LED light source (DC2200, Thorlabs) and filtered through a TRITC/Cy3/TagRFP/Alexa Fluor 546 filter set (39004, Chroma Technology Corporation, Rockingham, VT) allows us to visualize the fluorescently labeled polarity-marked microtubules. We detect the displacement of the bead from the trap center using quadrant photodiode-based (QPD, QP45-Q-HVSD, First Sensor, AG, Berlin, Germany) back focal plane detection (49).

We used power spectral density analysis on the position of the trapped bead to calibrate the trap stiffness for each bead (0.09–0.1 pN/nm) (50). In brief, we converted the QPD voltage into the displacement of the bead from the trap center using the position detection sensitivity factor (β in V/μm) obtained from a stuck bead calibration (50). Then, we divided the measured voltage signal β and multiplied the resultant by trap stiffness to get force traces.
bright GMPCPP minus-ends to identify the microtubule polarity. We oscillated the nanopositioning stage in a triangle wave pattern (2 μm amplitude, 0.2 Hz) with a custom-written LabVIEW virtual instrument (NI, Austin, TX). Given the trap stiffness of 0.09–0.1 pN/nm, this triangle wave pattern stage movement would correspond to a constant loading rate force ramp of 145–160 pN/s, assuming negligible stretching in the MTBD-antibody-biotin-streptavidin-bead system. However, we found that the actual loading rate was 40 pN/s when considering the system’s overall compliance (supporting material). This technique effectively measures force-induced dissociation under a full spectrum of applied loads. We observed binding/unbinding events for approximately 30% of MTBD-coated beads, suggesting that most detected interactions were due to single-molecule binding and unbinding events (52, 53). We tested 10–15 beads in all cases. We found that some beads showed nonspecific binding to the coverslip, and that some showed nonspecific binding to the coverslip.

Analysis of optical tweezers-based force-dependent dissociation experiments

We acquired the optical tweezers data (force traces) at 1 kHz using an FPGA (PXI7854R, NI) and custom-written LabView virtual instruments. We filtered the data using a Savitzky-Golay filter (polynomial order 4 and frame length 61 ms) (54). When a microtubule-binding event occurred, the moving stage pulled the bead from the trap’s center, causing a peak in the force trace.

We distinguished binding/unbinding events from the noise using custom-written MATLAB (The MathWorks, Natick, MA) scripts. To distinguish binding events from the noise, we calculated the standard deviation of each force trace and took an initial pass at identifying binding/unbinding events by finding the peaks in the force spectroscopy data that are greater than four standard deviations above the mean force. This first pass overestimates the noise because the calculation includes the signal. Therefore, we excluded the identified peaks from the time-series data and calculated the standard deviation of the reduced data, i.e., the standard deviation of the noise. We took a second pass at identifying binding/unbinding events by finding the peaks that were at least 4.5 standard deviations above or below the mean. We repeated this process iteratively until we found no additional peaks of 4.5 standard deviations above the noise. We set the force values of 4.5 standard deviations above and below the mean as the upper and lower thresholds, respectively, and we take any data that surpass these thresholds as an identified binding event.

We calculated the bound time for each identified event as the interval between the time that the force trace last crossed the 1 standard deviation above or below the mean level (the upper and lower transversal forces, respectively) before the peak data point and the time that the force trace next passed the transversal force after the peak data point (Fig. S4 C in the supporting material, for example, peaks with upper and lower threshold forces, and upper and lower transversal forces shown). We used the peak force as the unbinding force associated with the event.

We further analyzed each peak to identify spurious events that made it through our peak identification process. We excluded data points that fall into one of two categories, low-force long-duration and high-force double-peak events (Fig. S4 C), because neither of these types of events represents single MTBD-microtubule unbinding events (see supporting material for details).

We plotted the cumulative distribution function (CDF) of the bound times for the MTBDs in the force-dependent dissociation assays and fit the CDFs with an exponential function (55):

\[
CDF(t) = 1 - e^{-\frac{t}{\tau}} ,
\]

where \( t \) is the bound time for each unbind event, \( t_0 \) is the minimum resolvable binding time (56), and \( \tau \) is the characteristic force-dependent unbinding time. We performed Kolmogorov-Smirnov (K-S) tests on the underlying bound time distributions to evaluate whether the collected samples could have been drawn from the same (but unknown) probability distribution (in this case, the distribution of unbinding time). A K-S test returning a \( p > 0.05 \) fails to support the null hypothesis that the sample data come from different underlying distributions; a \( p < 0.05 \) suggests that the sample data likely come from different underlying distributions.

RESULTS

To investigate the role of long-range electrostatic interactions in regulating the binding affinity of the dynein MTBD for microtubules, we performed binding affinity and dissociation assays using an SRS domain high-binding affinity locked mouse cytoplasmic dynein MTBD (Materials and methods) (22). We compared results from the WT MTBD to MTBD mutants with charge-altering amino acid substitutions that were remote from the MTBD-microtubule-binding interface under multiple charge screening conditions (Materials and methods) (22). We made these changes far from the microtubule-binding interface (D3402A and E3320A, Fig. 1 B) to alter long-range electrostatic interactions (33) but not affect short-range interactions (salt bridges and hydrogen bonds) at the binding interface (28), alter dynamic salt bridges (24) or cause structural changes to the MTBD.

Point mutations can alter the structural stability of proteins depending on the location and type of the mutation. Such structural changes could affect the binding affinity of a protein for its partner biomolecule or ligand (57, 58) in ways that are not strongly correlated with long-range electrostatic interactions. Therefore, we assessed the effect of the mutation on the stability of the D3402A and E3320A mutants using circular dichroism (Fig. S2 A). We found no significant difference between the α-helical content of the WT and the D3402A mutant MTBDs (\( p > 0.05 \), Fig. S2 B, two-sample t-test). However, the α-helical content E3320A was reduced 0.4-fold compared with WT MTBD (\( p < 0.001 \), Fig. S2 B, two-sample t-test).

We further assessed the impact of the D3402A and E3320A charge-altering mutations on MTBD protein structure by performing stability calculations using SDM (40) and DynaMut (39). In accordance with the CD results (Fig. S2 A), both algorithms predicted that the E3320A mutation should destabilize and that the D3402A mutation should stabilize the MTBD’s structure (see Table S1; Fig. S2 B, and accompanying text in the supporting material for details).

Together, the CD and computational algorithms both predicted that the E3320A mutation caused significant structural differences. It was impossible to deconvolve any changes in binding affinity due to the E3320A mutation from differences due to the long-range electrostatic changes. Therefore, we only used the WT and D3402A mutant proteins in our experiments to probe the effect of long-range electrostatic interactions on dynein MTBD-microtubule binding.
**The binding affinity of D3402A mutant MTBDs for the microtubule is higher than WT MTBDs**

We measured the $K_d$ of the MTBDs and microtubules using MST (Materials and methods) to quantify the effect of long-range electrostatic interactions on the binding affinities of MTBD for microtubules. We titrated increasing concentrations of microtubules into a solution containing 50 nM fluorescently labeled MTBD, acquired MST traces, and found that the normalized fluorescence of MST traces decayed slower and to a higher steady-state normalized fluorescence with increasing concentration of microtubules (Fig. 1 C). These data suggest that MTBD bound to microtubules has lower thermophoretic mobility than free MTBD.

We fit the MST traces to a linearized model of MST (Materials and methods) to calculate the fraction of MTBD bound to microtubules and plotted the results as a function of microtubule concentration (Fig. 1 D). Then, we fit Hill-Langmuir binding curves (Materials and methods) for the WT and the D3402A mutant, and we found that $K_d$ is 11-fold higher ($p < 0.0001$, two-sample $t$-test) for WT than for D3402A MTBD constructs ($K_d = 5.11 \pm 0.33 \mu$M and $K_d = 0.46 \pm 0.06 \mu$M, mean $\pm$ standard error of the mean, $N = 3$ for WT and $N = 4$ for the D3402A mutant) in the absence of additional salt (Fig. 1 D).

The MST results show that the binding affinity of the D3402A mutant MTBDs for the microtubule is higher than WT MTBDs, suggesting that long-range electrostatic interactions either guide the MTBD’s binding to the microtubule, stabilize the MTBD-microtubule bound structure, or both.

**The force-dependent unbinding rate of D3402A mutant MTBDs from microtubules is slower than WT MTBDs**

External forces can regulate the biophysical properties of dynein by altering the binding affinity of MTBD toward microtubules depending on the direction of applied force (53,59). We measured the force magnitude and direction-dependent MTBD-microtubule dissociation rate using optical tweezers (Materials and methods) to study the effect of long-range electrostatic interactions on the force-dependent MTBD-microtubule interactions. We conjugated the MTBD to beads (Materials and methods) and presented them to polarity-marked microtubules (Materials and methods) by oscillating the stage position (triangle wave of displacement) with respect to the optical trap (Materials and methods; Fig. 2 A). We captured time-series force spectroscopy data of MTBD-microtubule interactions (Fig. 2 B and 2 C), binned binding events as occurring under hindering (microtubule plus-end directed) and assisting (microtubule minus-end directed) loads (Fig. 2 A), and found the bound time and unbinding force for each binding event (Fig. 2 C; Materials and methods).

We plotted the cumulative distribution of single-molecule binding event bound times for the WT MTBD and the D3402A mutant MTBD, and we fit the data to exponential CDFs (Eq. 2) for events subject to assisting and hindering loads (Fig. S5 in the supporting material). We found that, while the binding time was a function of the mutation (Fig. S5), the CDFs were not statistically different from each other ($p > 0.05$ in all cases, two-sample K-S test) as a function of load direction. These results indicate that the force-dependent unbinding of the MTBD from the microtubule is symmetric with respect to the direction of load, which is consistent with previous reports (53). Therefore, we pooled the assisting and hindering data for further analysis.

We compared the pooled distributions of bound time for the WT and D3402A mutant MTBD-microtubule force-dependent dissociation events, and we found that they came from statistically distinct populations (Fig. 2 D, $p < 0.001$, two-sample K-S test). We fit the exponential CDF (Eq. 2) to the cumulative distribution of bound times for the WT MTBD and D3402A mutant MTBD-microtubule force-dependent dissociation events (Fig. 2 D). We found that the characteristic bound time, $\tau$, was approximately twofold larger for the D3402A mutant than WT MTBDs (Fig. 2 D).

The force-dependent results show that the unbinding rate of D3402A mutant MTBDs from microtubules is slower than WT MTBDs, suggesting that long-range electrostatic interactions propagate through the MTBD and stabilize short-range salt bridges at the MTBD-microtubule-binding interface.

**Increasing the ionic strength of the solution differentially affects the MST-derived $K_d$ and the optical tweezers-determined force-dependent unbinding rate**

We repeated the MST and optical tweezers assays on the WT and the D3402A mutant MTBDs in the presence of additional salt to decrease the Debye length in the solvent from approximately 0.8 nm for no additional salt to 0.6 and 0.4 nm for an additional 100 and 300 mM KCl, respectively (Eq. 1). Additional salt increases the charge screening of long-range electrostatic interactions that act through the solvent.

We found that the addition of 100 mM KCl did not have a significant effect on the $K_d$ for WT MTBDs ($p = 0.57$, two-sample $t$-test, $N = 3$ and $N = 2$ for 0 and 100 mM KCl, respectively, Fig. 3 A), as measured using MST (Materials and methods). However, the addition of 100 mM KCl had a much greater (a 12-fold increase), highly significant ($p < 0.0001$, two-sample $t$-test, $N = 4$ and $N = 2$ for 0 and 100 mM KCl, respectively, Fig. 3 A) effect on the $K_d$ for the D3402A mutant MTBDs than WT MTBDs. The additional salt reduced the 11-fold difference in $K_d$
The characteristic unbinding time was a constant loading rate of approximately 40 pN/s (Materials and methods and supporting material). The CDFs were statistically different from each other mutant (Fig. 3).

The CDFs were statistically different from each other mutant (Fig. 3). The additional salt at 300 mM KCl further masked the differences between the $K_d$ for WT MTBDs and the D3402A mutant MTBDs (13.3 ± 3.7 μM and 14.7 ± 6.4 μM, $p = 0.87$, two-sample $t$-test, $N = 2$ in both cases, Fig. 3 A).

We also found that the addition of salt (both 100 and 300 mM KCl) had a statistically negligible effect ($p > 0.05$, two-sample K-S tests, Table S3) on the cumulative distribution of bound times for WT MTBDs when subject to increasing load (approximately 40 pN/s, supporting material) by examining the optical tweezers. In addition, the addition of 100 mM KCl had a statistically negligible effect ($p = 0.77$, two-sample K-S test, Table S3) on the cumulative distribution of bound times for D3402A mutant MTBDs when subject to increasing salt. Furthermore, the addition of 300 mM KCl restored the cumulative distribution of bound times for D3402A mutant MTBDs to being statistically indistinguishable from the WT MTBDs ($p > 0.05$, two-sample K-S tests, Table S3).
We also fit the exponential CDF (Eq. 2) to the cumulative distribution of bound times for the WT MTBD and D3402A mutant MTBD-microtubule force-dependent dissociation events in 100 and 300 mM KCl additional salt. We found that the characteristic bound time, $\tau$, was nearly 1.75-fold larger (approximately 0.14 s, based on the mean of $\tau$ for 0 and 100 mM additional KCl, Fig. 3B) for the D3402A mutant MTBDs at no and low (100 mM) additional salt compared with WT MTBDs (approximately 0.08 s, based on the mean of $\tau$ for all three conditions, Fig. 3B). In addition, we found that the highest (300 mM) additional salt concentration essentially masked the differences between the characteristic bound time of D3402A and WT MTBDs (0.8-fold, but $p > 0.05$ from all two-sample K-S tests, Table S3).

Together, these results show that $K_d$ and force-dependent unbinding rate differentially depend on the ionic strength of the solution. They also suggest that long-range electrostatic interactions are more strongly attenuated by salt at thermodynamic binding equilibrium than when bound to the microtubule.

**DISCUSSION**

In this study, we investigated whether long-range electrostatic interactions, i.e., electrostatic interactions between charged amino acids not at the immediate binding interface, play a significant role in binding cytoplasmic dynein-microtubule binding domains locked into the high-binding affinity state to microtubules. We used both site-directed mutagenesis (to change a charge remote from the binding interface, Fig. 1B, but did not change the structure, Fig. S2) and salt-induced charge screening (to change the Debye length, Eq. 1) to probe these effects. We found that long-range electrostatic interactions do play a significant role in the binding affinity by affecting both the $K_d$ and the unbinding rate, $k_{off}$.

Using MST, we found that the $K_d$ of the WT MTBD was more than 10-fold higher than the positive charge-neutralizing D3402A mutant (Fig. 1D). This result strongly suggests that long-range electrostatic interactions play a critical role in the binding affinity, as the D3402A mutation is 2.4 nm from the binding interface.

We further probed the effect of long-range electrostatic interactions by increasing the electrolyte concentration in the solution. We found that the addition of 100 mM KCl did not significantly affect the $K_d$ of the WT MTBD (Fig. 3A), but it restored the WT affinity to the D3402A mutant MTBD (Fig. 3A). The further addition of salt to 300 mM of additional KCl masked the affinity difference of both MTBDs toward microtubules.

Together, the MST results must be understood in the context of the differences between the effects of charge screening on the electric field lines that pass through the solution and those that pass through the solvent-excluded volume of the protein. Debye-Hückel theory (60) shows that the electric potential due to charged amino acid residues at a protein surface decreases by a factor of 1/e (approximately 63% attenuated) for each Debye length into the solvent (Eq. 1). In both cells and biological buffers with an electrolyte concentration of approximately 150 mM, the Debye length is approximately 0.8 nm (Eq. 1). Thus, the strength of the electrostatic field lines between aspartic acid 3402 in the MTBD and the microtubule surface that pass through the solvent are attenuated by about 95%. However, many of the electrostatic field lines between D3402 and the microtubule surface pass through the solvent-excluded protein-filled regions of space occupied by the rest of the MTBD. Debye-Hückel theory does not apply in these regions. Thus the magnitude of the electric field strength is unattenuated by charge screening. Moreover, the electric field strength, $E = \frac{q}{2\pi \varepsilon_0 r^2}$, where $q$ is the charge on the amino acid, $r$ is the distance between the charged amino acid and the microtubule, and $\varepsilon_0$ is the electrical permittivity of free space, is stronger in protein-filled regions of space than solvent-filled regions of space because the dielectric constant, $\kappa$, is lower in the protein (often assumed to be 4) than in the solvent, which has $\kappa = 80$. 

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**FIGURE 3** Solution ionic strength differentially affects the dissociation constant and characteristic bound times of WT and D3402A mutant MTBDs on microtubules. (A) The dissociation constant ($K_d$) for the WT and D3402A mutant MTBDs obtained by fitting Hill-Langmuir binding curves to the fraction bound for each KCl concentration case in MST experiments. Error bars represent the standard error of the mean of the MST curves to the fraction bound for each KCl concentration case in MST experiments, similar to Fig. 2B. (B) Characteristic force-dependent unbinding time, $\tau$, obtained from the fits of cumulative distributions of bound times data to Eq. 2 with $t_0 = 0.065$ s. Error bars represent standard errors in both panels. MTBD construct and additional salt concentrations legends and labels apply to both panels.

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**Table S3**
Thus, despite the relatively high concentration of electrolytes, long-range electrostatic interactions can significantly affect binding affinity.

Using optical tweezers to characterize the force-dependent dissociation of the MTBDs from microtubules, we found that the characteristic force-dependent unbinding time when subject to a constantly increasing load, \( \tau \), of the positive charge-neutralizing D3402A mutant was approximately 1.75-fold higher than the WT MTBD (Fig. 2 D). We further probed the effect of long-range electrostatic interactions by increasing the electrolyte concentration in solution, and we found that neither the addition of 100 nor 300 mM KCl strongly affected \( \tau \) of the WT MTBD (Fig. 3 B). Only the addition of 300 mM KCl, but not 100 mM KCl, restored the WT value of \( \tau \) to the D3402A mutant MTBD (Fig. 3 B). These results suggest that increasing the long-range electrostatic forces significantly stabilized the MTBD-microtubule bound structure. Moreover, because it took a large amount of added salt to screen the increased long-range electrostatic interactions due to the D3402A mutation, these results suggest that long-range electrostatic interactions in the MTBD-microtubule interface primarily act through regions of space that are minimally solvent accessible, i.e., through the globular MTBD domain. These results are consistent with other systems in which long-range interactions are essential for binding, e.g., TEM1 \( \beta \)-lactamase and its protein inhibitor BLIP (63) and kinesin and microtubules (64).

Taken together, the MST and force-dependent dissociation results show that the charge screening affects the equilibrium \( K_d \) more strongly, and with more sensitivity (lower salt is necessary) than the unbinding kinetics \( k_{\text{off}} \), which scales like \( 1/\tau \) of MTBD-microtubule interactions. This suggests that long-range electrostatic interactions are particularly important to aspects of biomolecular bonding systems that are not highly susceptible to charge screening by soluble ions. For example, the solvent and soluble ions are excluded from the MTBD-microtubule interface when the MTBD is bound to the microtubule, which reduces the Debye effect (Fig. 4 A, left) and thus the relative insensitivity of the unbinding kinetics \( (k_{\text{off}}) \) to additional salt. However, the solvent and soluble ions fill the space between the MTBD and the microtubule when the MTBD is not bound to the microtubule, which screens charges due to the Debye effect (Fig. 4 A, right) and thus the relative sensitivity of the equilibrium \( K_d \) to additional salt.

Moreover, this model has implications for the molecular details of dynein motility and processivity. It suggests that, as an MTBD moves through the electrostatic binding funnel, which guides and aligns MTBDs to the binding interface on the microtubule (33,34,64,65) as well as other biomacromolecular interactions (66–70), it displaces more solvent and reduces the Debye effect, effectively reducing the attenuation of the electrostatic field strength and increasing the electrostatic forces. Thus, this model suggests that a positive physical force feedback mechanism for biomolecular binding driven by long-range electrostatic interactions could be important to the molecular mechanism of processivity of dynein motor proteins.

The observed significant decrease in the \( K_d = \frac{k_{\text{on}}}{k_{\text{off}}} \) upon changing the charge of an amino acid remote from the binding interface suggests that either the binding kinetics, \( k_{\text{on}} \), increased or the unbinding kinetics, \( k_{\text{off}} \), decreased, or both. The observed increase in \( \tau \) suggests a decrease in \( k_{\text{off}} \).
because the characteristic bound timescales like $k_{\text{off}}^{-1}$. However, the magnitude of the increase in $\tau$ (1.75-fold) is unlikely to explain the magnitude of the decrease in $K_d$ (11-fold). This further suggests that the change in the long-range electrostatic interactions induced by the charge-altering mutation remote from the binding interface increased $k_{\text{on}}$, and thus the MTBD’s microtubule-binding funnel.

Altogether, our results support our previous computational studies (33,34), which also highlighted the significant role of long-range electrostatic interactions in guiding the MTBD binding to microtubules by providing an electrostatic binding funnel. Similar long-range electrostatic interaction-guided regulation mechanisms were reported earlier in the case of kinesin motors (64,65,71). Together with the results from previous experimental work detailing how the short-range electrostatic interactions that constitute surface charge complementarity at the MTBD-microtubule-binding interface (22,28,30) and specific, dynamic salt bridges within the MTBD (24) underlie the microtubule-binding affinity, our results help build a more complete picture of how electrostatics govern the molecular mechanics of dynein motility. We propose that long-range electrostatic interactions, short-range electrostatic interactions, and dynamic salt bridges significantly contribute to various aspects of the binding affinity mechanism as a function of the distance the MTBD is from the microtubule. The net charge of the MTBD, which includes charges at and far from the binding pocket, guides the initial binding process (Fig. 4B). The distribution of charge on the MTBD, including long-range interactions remote from the binding interface, can apply electrostatic torques to the MTBD that may be particularly important to reorienting (33) the MTBD as it approaches the microtubules. As dynein’s MTBD achieves a favorable binding pose, our model suggests that the binding kinetics accelerate due to solvent exclusion induced electrostatic charge screening reduction, and, once bound, the short-range electrostatic interactions that “key” the alignment (33) and dynamic salt bridges that regulate affinity can be further stabilized or destabilized by long-range interactions within the MTBD (Fig. 4B). All the effects described in this model are further regulated by the role that the negatively charged, intrinsically disordered, highly posttranslationally modified, C-terminal tail of tubulin plays in guiding the binding process and regulating the bound complex (34).

It is possible that the predicted MTBD structural stabilization upon mutation (Table S1) could also contribute to the binding behavior that we observed and attributed to the effects on long-range electrostatic interactions, above. We found that DynaMut and SDM predicted that the D3402A mutation stabilized the folding free energy ($\Delta G$) by 0.15 and 1.24 kcal/mol, respectively. These are two and one orders of magnitude smaller than the calculated change in the electrostatic binding energy (10 kcal/mol) upon mutation (33). Moreover, the binding constants we probed in this work go as the exponential of $\Delta G$, and we did not see significant secondary structural differences (Figure S2) for the WT and D3402A mutant. Together, these calculations and observations suggest that the changes we observed in binding behavior due to the mutation predominantly arise from long-range electrostatic effects.

Beyond the role of electrostatic interactions, acidic amino acid residues, in particular aspartic acid, have been shown to be the primary feature of proteins that stabilizes and solubilizes them in the presence of the high salt concentrations experienced by halophiles, which are extremophiles living in environments with more than five times the salinity of sea water (72). Aspartic acids provide favorable sites for water molecules in the ordered hydration layer to hydrogen bond with the protein surface at higher salt concentration, leading to increased hydration shell stability of aspartic acid-rich protein surfaces (73,74). By mutating aspartic acid 3402 to an alanine, we could have made the MTBD more susceptible to increased salt concentration, although the additional 300 mM (at the most) KCl is significantly lower than the 2.5–5 M salt concentrations experienced by halophiles. This effect could help explain the difference in the change in the $K_d$ values in the D3402A mutant MTBD as compared with that in the WT MTBD when going from no salt to 100 mM salt (Fig. 3A). Along the same lines, the microtubule-binding dynamics of the WT MTBD were significantly less susceptible to the increased salt concentration, as all three conditions considered showed similar unbinding rates (Fig. 3B). This analysis reiterates that the physical effects of the amino acids in proteins act at multiple levels, with multiple modes, and across multiple scales.

These results, and the models they suggest, have significant implications on our understanding of processivity in the dynein family of motor proteins. All dynein family members likely rely on specific short-range electrostatic interactions and the ability to change these interactions as a function of mechanochemo cycle state, coiled-coil stalk registration, and MTBD structural conformation. However, our results suggest nonspecific long-range electrostatic interactions that provide bound-state stabilization may be more prominent in processive cytoplasmic dyneins than axonemal dyneins. Future work on the motility of full-length dynein motor proteins with long-range electrostatic interaction-altering mutations under various salt conditions would be necessary to investigate these suggestions. Moreover, tuning the long-range electrostatic interactions without altering the binding interface residues could be a useful strategy to engineer the motor proteins with different processivities. Finally, our results also suggest that similar regulation mechanisms may be important in other motor proteins and for other protein-protein interactions in general.

**SUPPORTING MATERIAL**

Supporting material can be found online at https://doi.org/10.1016/j.bpj.2022.03.029.
AUTHOR CONTRIBUTIONS

J.A. designed the research. A. Pabbathi, L.C., M.S., and J.E. performed the research. A.G., A. Paul, and J.A. contributed the analytic tools. A. Pabbathi, L.C., S.G., A. Paul, A.G., and J.A. analyzed the data. A. Pabbathi and J.A. wrote the manuscript.

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SUPPORTING CITATIONS

References (75–77) appear in the supporting material.

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