Unconventional Processive Mechanics of Non-muscle Myosin IIB

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Proper tension maintenance in the cytoskeleton is essential for regulated cell polarity, cell motility, and division. Non-muscle myosin IIB (NMIIB) generates tension along actin filaments in many cell types, including neuronal, cardiac, and smooth muscle cells. Using a three-bead optical trapping assay, we recorded NMIIB interactions with actin filaments to determine if a NMIIB dimer cycles along an actin filament in a processive manner. Our results show that NMIIB is the first myosin II to exhibit evidence of processive stepping behavior. Analysis of these data reveals a forward displacement of 5.4 nm and, surprisingly, frequent backward steps of −5.9 nm. Processive stepping along the long pitch helix of actin may provide a mechanism for disassembly of fascin-actin bundles. Forward steps and detachment are weakly force-dependent at all forces, consistent with rate-limiting and force-dependent ADP release. However, backward steps are nearly force-independent. Our data support a model in which NMIIB can readily move in both directions at stall, which may be important for a general regulator of cytoskeleton tension.

EXPERIMENTAL PROCEDURES

Protein—The chicken non-muscle IIB HMM (residues 1–1228) construct (GenBank™ accession number M93676, no splice insert) in pFastBac was a generous gift of Drs. Sellers and Adelstein (20). We used overlap extension PCR to truncate NMIIB after residue 1228 and insert a three-amino acid linker (AAE), green fluorescent protein tag, and FLAG tag (DYKDDDK) for purification (see supplemental Fig. S1). This construct lacks the sequence determinants for thick filament formation. Virus containing both the regulatory and essential light chains (BLC virus) was obtained from Dr. Adelstein. NMIIB was purified from either Si9 or Hi5 insect cells as described previously.
NMIIB Was Dialyzed against Assay Buffer (AB; 25 mM KCl, 4 mM MgCl₂, 20 mM imidazole·HCl, pH 7.5, 0.5 mM EDTA, 1 mM EGTA, 0.5% (w/v) Igepal, 7% (w/v) sucrose, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 µM dithiothreitol, 2 mM ATP (Calbiochem)). Cells were sonicated to achieve complete lysis. ATP was added to the lysate to bring the concentration to 3 mM, and the lysate was sonicated for 1 h at 4 °C. The lysate was centrifuged for 10 min at 4000 rpm to pellet cell debris. The supernatant was applied to a disposable column (Promega) and washed with 30 ml of Wash Buffer (150 mM KCl, 20 mM imidazole·HCl, pH 7.5, 5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 3 mM ATP, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) under gravity flow. The column was washed with 20 ml of Kinase Reaction Buffer (25 mM Tris·HCl, pH 8.0, 5 mM MgCl₂, 1.5 mM CaCl₂, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol). NMIIB was phosphorylated overnight at 4 °C on the column in a 2-ml volume of 4 µg/ml calmodulin, 4 µg/ml myosin light chain kinase (prepared according to Adelstein and Klee (22) and 2 mM ATP (Calbiochem)) in kinase reaction buffer. The column was washed again with 30 ml of Wash Buffer. NMIIB was eluted from the column using 2 ml of Wash Buffer with 0.2 mg/ml FLAG peptide (Sigma). The resin was incubated with FLAG peptide for 1 h, and then the eluate, containing NMIIB, was collected. Eluted NMIIB was dialyzed first against Assay Buffer (AB; 25 mM KCl, 25 mM imidazole·HCl, pH 7.5, 1 mM EGTA, 4 mM MgCl₂, 20 mM β-mercaptoethanol) and second against AB with 50% (w/v) glycerol and then stored at −20 °C. Light chain phosphorylation was verified by Tris-glycine gel electrophoresis (23).

Sea urchin calmodulin (24), chicken skeletal muscle actin (25), biotinylated (1:10) actin stabilized with tetramethylrhodamine-phalloidin, 1-µm neutrophilic beads (0.02% solids) (30), and 1 µM phalloidin (Sigma). At the myosin densities used in these experiments, −1 in 10 platforms showed motor activity (93 platforms were tested, 7 exhibited staircases activity, and 3 showed binding events that were primarily non-processive, although some processive events were still observed). The probability that a surface platform with binding events has multiple motors is 5%. Given that probability, the binomial probability that finding 7 or more platforms of 10 with staircases due to two motors is 1 × 10−7. Reported NMIIB activity is not the result of stage drift because such drift is 70-fold slower than our observed velocity (maximal drift of surface-immobilized reporter beads is 0.3 nm/s, versus 21 nm/s for our observed low load velocities). Moreover, stage drift cannot account for the kinetic features that we observe, including an approach to stall around 2 pN (see Fig. 6). We used a custom-built optical trapping/TIRF microscope, modeled after similar instruments described previously (29, 31). The instrument generated multiple traps by rapidly chopping (20 kHz) trap position using a pair of orthogonal acoustooptical deflectors (Intraaction). Bead positions were detected with back focal plane detection (31), using two fiber-coupled laser diodes (785 and 850 nm) as probe beams and two spectrally separated duolateral position-sensitive diodes placed in planes conjugate to the back focal plane of the condenser. Detector responses were calibrated by raster-scanning the bead through the detection area and calculating a 5th order, two-dimensional polynomial response function (31). Bead positions were low pass antialias-filtered at 1 kHz with a custom 4-pole Bessel filter and digitized at 2 kHz. Measured trap stiffnesses were 0.04 pN/nm.

Gliding Filament Assays—All assays were performed at 22 °C in flow cells constructed from a glass slide, two pieces of double-sided tape, and a nitrocellulose-coated coverslip. Flow cells were incubated with 0.05 mg/ml green fluorescent protein antibodies (Qiagen, 0.05 mg/ml in phosphate-buffered saline, 2 min), followed by a bovine serum albumin block (1 mg/ml in AB; 2 min). NMIIB was added to the flow cell at a concentration of 450 nM and incubated for 2 min. The flow cell was rinsed with 10 µl of AB and then incubated for 2 min with 100 nM F-actin in AB, stabilized with tetramethylrhodamine-phalloidin (Sigma). The flow cell was washed again with 10 µl of AB. Finally, Motility Buffer was added, and actin filaments were visualized on a Zeiss Axiovert 200 microscope with an Andor Luca camera. Motility Buffer contained the indicated amount of ATP, 2 mM free Mg²⁺, 0.086 mg/ml glucose oxidase, 0.014 mg/ml catalase, and 0.09 mg/ml glucose and 1 µM dithiothreitol in AB. Bundled motility assays were performed similarly using a 2 µM dilution of fascin-actin bundles incubated for 2 min. In assays with rabbit skeletal muscle HMM (a generous gift of Roger Cooke), the myosin was applied directly to the nitrocellulose coverslip, omitting the antibody and prior bovine serum albumin block step.

Three-bead Trapping Assay—We used previously described optical trapping protocols and reagents (28) in a three-bead geometry (29). Briefly, a flow cell made with bead-coated coverslips (1.5-µm diameter silica, Bangs Laboratories) was incubated with green fluorescent protein antibodies, followed by bovine serum albumin and NMIIB (0.8–2.7 nM) The flow cell was washed with AB, and Motility Buffer was flowed in. Motility Buffer contained 2 mM ATP (or 10 µM ATP with free Mg²⁺ concentration adjusted to 2 mM), 0.086 mg/ml glucose oxidase, 0.014 mg/ml catalase, 0.09 mg/ml glucose, 1 nM biotinylated (1:10) actin stabilized with tetramethylrhodamine-phalloidin, 1-µm neutrophilic beads (0.02% solids) (30), and 1 µM phalloidin (Sigma). At the myosin densities used in these experiments, −1 in 10 platforms showed motor activity (93 platforms were tested, 7 exhibited staircases activity, and 3 showed binding events that were primarily non-processive, although some processive events were still observed). The probability that a surface platform with binding events has multiple motors is 5%. Given that probability, the binomial probability that finding 7 or more platforms of 10 with staircases due to two motors is 1 × 10−7. Reported NMIIB activity is not the result of stage drift because such drift is 70-fold slower than our observed velocity (maximal drift of surface-immobilized reporter beads is 0.3 nm/s, versus 21 nm/s for our observed low load velocities). Moreover, stage drift cannot account for the kinetic features that we observe, including an approach to stall around 2 pN (see Fig. 6). We used a custom-built optical trapping/TIRF microscope, modeled after similar instruments described previously (29, 31). The instrument generated multiple traps by rapidly chopping (20 kHz) trap position using a pair of orthogonal acoustooptical deflectors (Intraaction). Bead positions were detected with back focal plane detection (31), using two fiber-coupled laser diodes (785 and 850 nm) as probe beams and two spectrally separated duolateral position-sensitive diodes placed in planes conjugate to the back focal plane of the condenser. Detector responses were calibrated by raster-scanning the bead through the detection area and calculating a 5th order, two-dimensional polynomial response function (31). Bead positions were low pass antialias-filtered at 1 kHz with a custom 4-pole Bessel filter and digitized at 2 kHz. Measured trap stiffnesses were 0.04 pN/nm.

Data Analysis—Steps were detected using the step finder algorithm (32). Dwell times less than 0.01 s were filtered from the force dependence analysis. The forward step probability (P(f)) is calculated as follows, P(f) = n(f)/{n(f) + n₀(f) + nₜ(f)}, where n(f) is the number of forward steps in a force bin (n₀(f) and nₜ(f) are the number of backward steps and detachment events, respectively), and (n(f) + n₀(f) + nₜ(f)) is the total number of steps and detachments in the same force bin. Backward step and detachment probabilities as a function of force are calculated in an analogous manner. Rates are calculated by multiplying the partitioning probabilities (see Fig. 6B) by the force-dependent stepping rate (see Fig. 6A, inset) at the corresponding force.

Simulations—We set up simulated trapping staircases by choosing total step numbers at random from a distribution of
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experimental results. We then chose dwell times for each step by sampling randomly from the exponential function with a time constant matching our experimentally determined dwells (0.28 s) and created a vector that had a length determined by the sum of the dwell times in each staircase sampled at a rate of 2 kHz. Each point in that vector was then assigned a value by multiplying the index of the step corresponding to that moment in time by a fixed step height (4 ± 0.05 nm (S.D.), gaussian). The simulated data traces were then low pass-filtered to produce a corner frequency in the power spectrum corresponding to our experimental system. Simulated data traces were processed using a step finder algorithm (32). The resulting step size histogram and survivor function are shown in supplemental Fig. S2.

RESULTS AND DISCUSSION

An Unconventional Processive Stepping Pattern—We constructed and purified a dimer of non-muscle IIB (HMM), containing the motor domain, light chain binding domains, truncated coiled-coil tail, and green fluorescent protein. At saturating ATP, NMIIB propels actin at ~29 nm/s (supplemental Fig. S1) in gliding filament assays (33). This velocity is extremely slow, especially when compared with the velocity of smooth muscle myosin II (600 nm/s), which shares 80% sequence identity with NMIIB (34). Kovács et al. (18) showed that NMIIB can bind in a two headed conformation to neighboring actin monomers on the same protofilament and that ADP analogs release at different rates from the leading and trailing heads when both are bound to actin. These results led us to consider the possibility (also raised by Kovács et al. (18)) that NMIIB is processive. We attempted to visualize processive runs of NMIIB on immobilized actin filaments via TIRF microscopy but were unsuccessful (35). As we show below, this is most likely due to the short run length of NMIIB.

In order to address the question of processivity in NMIIB, we used a three-bead optical trapping assay. Fig. 1A shows several consecutive examples of an individual NMIIB motor interacting with an actin filament. The motor binds to actin, clamping some of the Brownian motion. The myosin then continues to move for several steps along the filament before releasing, generating a staircase pattern. A stepping pattern that begins with an initial displacement and is followed by subsequent displacements is indicative of processive stepping. In contrast, all other myosin IIs tested to date (skeletal muscle, smooth muscle, cardiac, and Dictostelium) will bind and produce a single power-stroke before detaching (15, 29, 36–38) (however, see also Kitamura et al. (19) for an unusual skeletal muscle mechanism). Although NMIIB is a slow motor, we find that stage drift in our instrument cannot account for our observed staircases. When we examine individual binding events in detail, we find frequent reverse steps of NMIIB (see the arrows in Fig. 1B). Although the binding events in the data traces appear to be the result of multiple processive steps, the individual steps can be difficult to discern by eye because of the small step size and the compliance in the actin dumbbell, which makes the data noisy. We therefore used a step finder algorithm to find the transitions between steps (32). This is an effective algorithm for accurately detecting steps as small as 4 nm in noisy data records, as shown in supplemental Fig. S2 and also Gennerich et al. (39). At low ATP concentrations (10 μM), we find that NMIIB moves more slowly in our trapping records (Fig. 1C; velocity = 3.2 ± 0.5 nm/s, n = 174 events), as expected for genuine motor motility as opposed to stage drift.

Once the start and end point of each step were determined, we obtained the step size distribution (Fig. 2A) of NMIIB. Because we see two prominent lobes, we fit the step size distribution to a sum of two gaussians. The more populated gaussian distribution is at 5.4 nm, and the smaller gaussian distribution is centered at -5.9 nm (S.D. = 0.1 nm). Intriguingly, the intermonomer distance along an actin protofilament (i.e. the long pitch helix) is 5.5 nm, suggesting that this myosin walks along a single actin protofilament in a tight spiral path with a 72-nm pitch. This behavior is quite unlike that of other processive myosins, which step along the 36-nm pseudohelical repeat of actin to avoid such tight spiraling (26, 30) (in unconstrained experiments, myosin V and VI slowly spiral around actin, with a pitch of 1000–2000 nm (40–42)). Interestingly, backsteps
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FIGURE 2. NMIIB steps along neighboring actin monomers in the proto-filament. A, a histogram of step sizes within processive runs at 2 mM ATP is shown with a fit to two gaussians (mean = 5.4 and -5.9 nm, S.E. = 0.1 and 0.1 nm, n = 2527 steps from two experiments). The larger peak represents forward steps, and the smaller peak represents backward steps. These step sizes correspond to the space between actin monomers along the actin protofilament. B, a histogram of step sizes within processive runs at 10 μM ATP is shown with a fit to a single gaussian (mean = 5.7 nm, S.E. = 0.1 nm, n = 507 steps from one experiment).

The dwell time distribution for all steps below 1 pN force can be fit to a single exponential decay (Fig. 3A), with a dwell time of 0.29 s at saturating ATP (2 mM). This single-exponential dwell time distribution indicates a single dominant rate-limiting transition in the NMIIB ATPase cycle. Furthermore, we can exclude a significant number of missing ATPase cycles with near zero step size. Such missed events would cause us to mistakenly observe dwells containing two consecutive ATPase cycles, which would lead to deviations from our observed single-exponential dwell times. At limiting ATP (10 μM), we again observe single exponential dwell time distributions, with an increased dwell time of 1.7 s (Fig. 3B). These data support the conclusion that NMIIB is a tightly coupled motor that hydrolyzes one ATP per step; in other words, the stepping rate is the ATPase rate (43, 44). If NMIIB hydrolyzed two ATPs per step, the dwell time distribution would be fit by a sigmoidal curve (Fig. 3B). If, instead, NMIIB could store the energy from a single ATP hydrolysis and parcel that energy out over multiple mechanical steps, we would observe a broader, multieponential dwell time distribution (not shown). As a processive, tightly coupled motor, each head of dimeric NMIIB must spend at least half of its time attached to actin (duty ratio >0.5).

We see an average processive run of 4.5 steps during each binding event (Fig. 4A), making NMIIB a weakly processive myosin compared with myosin V (40–60 steps) (45). Because backsteps are frequent with this motor, the distance traveled in a single processive run (the run length) is significantly shorter than 4.5 × 5.4 nm. Instead, we find a mean run length of ~12 nm, calculated from the start and end positions of each event and including the contribution from processive backsteps (Fig. 4B). These runs are far too short to be observed by standard TIRF microscopy methods, where motor runs of less than 200 nm are excluded from the analysis (35). However, we imagine that the run length of a NMIIB thick filament could be significantly longer in the cell if multiple motor dimers can engage the same actin filament.

The spiral path of NMIIB is unexpected because cytoskeletal motor proteins generally follow straight paths to avoid entangling their cargoes in the mesh of cytoskeletal filaments (41). However, no cargo transport roles have been identified for NMIIB. In a cell, NMIIB heads are likely to contact multiple actin filaments at each end of the bipolar thick filament. This cross-linking would effectively lock the thick filament in a relatively fixed orientation. If a NMIIB thick filament is locked in position while taking successive forward 5.5-nm steps along an actin protofilament, it should instead twist its actin track. In the context of an actin bundle, twisting filaments may be a mechanism for disassembling bundles by causing actin cross-linkers to detach from actin (46). In order to test this prediction, we immobilized NMIIB on a coverslip and applied fascin-bundled actin (parallel actin filaments) and saturating ATP. As the fascin-actin bundles translocate along the coverslip surface, individual filaments separate from the bundles and begin to move independently of each other (Fig. 5A), consistent with previous
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![Graph A](image1)

**Figure 4. Characterization of NMIIB processive stepping.** A, most binding events lead to multiple processive steps before detachment. A histogram of the number of forward and backward steps in a processive run shows an average of 4.5 steps/event ($n = 727$ events). B, a survivor function of run lengths (total distance traveled) shows an average run length of 12.0 ± 0.9 nm ($n = 464$ events). Dotted lines are lower and upper 95% confidence intervals, and the thick line shows a single exponential decay.

reports (47). This behavior was observed at all concentrations of NMIIB that caused filament translocation. We did not observe breaking of actin filaments by NMIIB over the time scale of this experiment. As a control, skeletal muscle myosin II HMM translocates fascin-actin bundles very long distances at saturating ATP without breaking or unbundling actin filaments (Fig. 5B), although it does break actin into short filaments at limiting ATP (Fig. 5C). Conventional skeletal myosin II is highly adapted for moving actin at high velocities and minimizing the interference between contracting catalytic heads. Presumably, at low ATP, many skeletal myosin catalytic heads can attach to actin. These heads interfere with the motility of other bound heads, resulting in breaking of actin filaments.

**An Unconventional Approach to Stall**—As NMIIB takes steps, it pulls the trapped beads further from their equilibrium positions, leading to increased force that resists forward motion (load). The force on the motor can affect the rates of mechanical transitions that occur during force-generating states of the myosin mechanochemical cycle. We therefore examined the effect of force on the dwell times of NMIIB steps. Fig. 6A shows dwell times and stepping rates plotted against the applied force at saturating ATP. Because NMIIB is apparently sensitive to even minor loads (unlike myosin V or myosin VI (30, 48)), we applied a simple transition state model with a single dominant load-dependent event, where the mean dwell time is given by $\tau = \tau_0 \exp(Fd/k_B T)$ (48, 49). Here, $k_B T = 4.09$ pN·nm, $\tau_0$ is the force-dependent dwell time at 0 pN force, and $d$ is the distance parameter (distance to the transition state). The distance parameter for all steps is 0.99 nm, meaning that NMIIB is weakly force-dependent. Our observed stepping rate at 0 pN load ($\tau_0 = 0.26$ s; $k_0 = 1/\tau_0 = 3.8$ s$^{-1}$) is significantly faster than the previously reported actin-activated Mg-ATPase rate for dimeric NMIIB (0.28 s$^{-1}$) or monomeric NMIIB (0.13 s$^{-1}$) (16, 21). We believe that our stepping rate is faster due to the presence of intramolecular strain when NMIIB takes processive steps. In the earlier studies, such strain may be difficult to detect if the motor cross-links two filaments at saturating actin. As we will explain below, our stepping rates are consistent with the measured ADP release rates of NMIIB experiencing intramolecular strain (18). As a further consistency check, we find that our stepping rates and step sizes are consistent with our gliding filament velocities because our calculated velocity from trapping records is 5.4 nm × 3.8 s$^{-1} = 21$ nm/s versus 29 nm/s for our gliding filament velocity. Note that we expect a similar motion prior to detachment events in the trap (see below), so detachments also propel actin filaments.

Although we have described the overall stepping kinetics as a function of load, we desire a more complete description of the kinetic pathways that explains the three fundamental events that we observe: a forward step, a backward step, or a detachment. To approach this problem, we separated dwell times into three categories based on whether they precede forward steps, backward steps, or detachments. For NMIIB, all three sets seem to be drawn from the same distribution, with the same mean dwell time and the same load dependence (longer dwell times at higher force) (supplemental Fig. S3). This type of behavior for motors has been previously ascribed to a model in which the dwell times for all types of events are dominated by the shortest dwell time (50). Essentially, the observed dwell time is a measure of the lifetime of a common state (see Fig. 6C, A), and the motor can exit that state via several mechanisms (i.e. forward stepping, backward stepping, or detachment (Fig. 6C)). The observed lifetime of NMIIB changes in response to load, as does the probability of taking a forward step, backward step, or detaching at any given load (Fig. 6B). Our observations can be best understood in terms of the simplest possible kinetic scheme shown in Fig. 6C. In this scheme, the motor dwells on the actin filament in a single dominant state and can exit that state by taking a forward step, backward step, or detaching, with an associated rate constant ($k_f$, $k_b$, and $k_d$) for each. We propose that any of these rates may be force-dependent. This scheme and the analysis that follows is identical to that for the single-state motor stepping model proposed by Kolomeisky et al.
Forward steps and detachments are force-dependent, but backward steps are nearly force-independent. A, observed rates for steps exhibit weak force dependence. Diamonds, mean ± S.D. (error bars) rates for 250 consecutive events along the force axis. Positive forces indicate forces that resist the motion of the myosin. The solid line shows the mean fit for a process with a single force-dependent step, \( \tau = \tau_0 \exp(Fd/k_BT) \) (a maximum likelihood ratio test does not support an additional force-independent rate parameter). Here, \( \tau_0 \) is the load-dependent dwell time at zero load, \( k_BT \) is thermal energy (4.09 pN•nm), and \( d \) is the distance to the transition state (60). For all steps, maximum likelihood parameter estimates are as follows: \( \tau_0 = 0.26 ± 0.01 \, \text{s} \) and \( d = 0.99 ± 0.09 \, \text{nm} \) (\( n = 2442 \) steps). All listed errors are S.E. from 500 bootstrap samples. Dotted lines are lower and upper 95% confidence intervals. Inset, stepping rate as a function of force. B, the partitioning of forward steps (red squares), backward steps (blue circles), and detachment events (black triangles) was calculated for each force bin (width = 0.2 pN) from the number of each type of event. See “Experimental Procedures” for calculating partition probabilities. C, kinetic scheme for interpreting the NMIIB dwell time data. M, NMIIB bound to actin; \( M_+ \), NMIIB after a complete cycle, having completed a forward step along actin; \( M_- \), NMIIB after a backward step; \( M_d \), NMIIB after detachment from actin. Forward stepping, backward stepping, and detachment occur at certain load-dependent rates defined as \( k_f(f) \), \( k_b(f) \), and \( k_d(f) \), respectively. D, the rates for forward stepping (symbols and colors as above) at each force were calculated by multiplying the partition probability of forward steps (Fig. 6B) by the observed rate at that force (Fig. 6A, inset). The force dependence of each rate was fit for a process with a single load-dependent step, \( k = k_0 \exp(-Fd/k_BT) \). For forward steps (red line), the fit parameters are as follows: \( k_f = 2.28 ± 0.12 \, \text{s}^{-1} \) and \( d_f = 1.6 ± 0.2 \, \text{nm} \) (\( n = 1284 \) steps). For backward steps (blue line), the fit parameters are as follows: \( k_b = 0.77 ± 0.04 \, \text{s}^{-1} \) and \( d_b = -0.35 ± 0.14 \, \text{nm} \) (\( n = 759 \) steps). The stall force (where forward and backward stepping rates are equal) for NMIIB is 2.2 pN. For detachment events (black line), the fit parameters are as follows: \( k_d = 1.00 ± 0.08 \, \text{s}^{-1} \) and \( d_d = 1.6 ± 0.2 \, \text{nm} \) (\( n = 587 \) events). All listed errors are S.E. obtained from performing the entire kinetic partitioning analysis on 200 bootstrapped samples.

FIGURE 6. Forward steps and detachments are force-dependent, but backward steps are nearly force-independent. A, observed rates for steps exhibit weak force dependence. Diamonds, mean ± S.D. (error bars) rates for 250 consecutive events along the force axis. Positive forces indicate forces that resist the motion of the myosin. The solid line shows the mean fit for a process with a single force-dependent step, \( \tau = \tau_0 \exp(Fd/k_BT) \) (a maximum likelihood ratio test does not support an additional force-independent rate parameter). Here, \( \tau_0 \) is the load-dependent dwell time at zero load, \( k_BT \) is thermal energy (4.09 pN•nm), and \( d \) is the distance to the transition state (60). For all steps, maximum likelihood parameter estimates are as follows: \( \tau_0 = 0.26 ± 0.01 \, \text{s} \) and \( d = 0.99 ± 0.09 \, \text{nm} \) (\( n = 2442 \) steps). All listed errors are S.E. from 500 bootstrap samples. Dotted lines are lower and upper 95% confidence intervals. Inset, stepping rate as a function of force. B, the partitioning of forward steps (red squares), backward steps (blue circles), and detachment events (black triangles) was calculated for each force bin (width = 0.2 pN) from the number of each type of event. See “Experimental Procedures” for calculating partition probabilities. C, kinetic scheme for interpreting the NMIIB dwell time data. M, NMIIB bound to actin; \( M_+ \), NMIIB after a complete cycle, having completed a forward step along actin; \( M_- \), NMIIB after a backward step; \( M_d \), NMIIB after detachment from actin. Forward stepping, backward stepping, and detachment occur at certain load-dependent rates defined as \( k_f(f) \), \( k_b(f) \), and \( k_d(f) \), respectively. D, the rates for forward stepping (symbols and colors as above) at each force were calculated by multiplying the partition probability of forward steps (Fig. 6B) by the observed rate at that force (Fig. 6A, inset). The force dependence of each rate was fit for a process with a single load-dependent step, \( k = k_0 \exp(-Fd/k_BT) \). For forward steps (red line), the fit parameters are as follows: \( k_f = 2.28 ± 0.12 \, \text{s}^{-1} \) and \( d_f = 1.6 ± 0.2 \, \text{nm} \) (\( n = 1284 \) steps). For backward steps (blue line), the fit parameters are as follows: \( k_b = 0.77 ± 0.04 \, \text{s}^{-1} \) and \( d_b = -0.35 ± 0.14 \, \text{nm} \) (\( n = 759 \) steps). The stall force (where forward and backward stepping rates are equal) for NMIIB is 2.2 pN. For detachment events (black line), the fit parameters are as follows: \( k_d = 1.00 ± 0.08 \, \text{s}^{-1} \) and \( d_d = 1.6 ± 0.2 \, \text{nm} \) (\( n = 587 \) events). All listed errors are S.E. obtained from performing the entire kinetic partitioning analysis on 200 bootstrapped samples.
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Having obtained these rate constants, we can now examine how our stepping behavior compares with the models proposed for NMIIB from solution kinetics. Our forward stepping rate at 0 pN load (2.3 s\(^{-1}\)) is only twice as fast as the previously measured value for ADP release from the trailing catalytic head experiencing intramolecular strain, 1.1 s\(^{-1}\) (Table 1 and supplemental Fig. S4) (18). Kovács et al. (18) also measured a rate for ADP release from a single attached catalytic head (0.27 s\(^{-1}\)) that is close to our detachment rate of 1.00 s\(^{-1}\). These results are consistent with a mechanism in which forward steps occur after release of ADP from the trailing head (experiencing a forward strain). Detachment occurs from an intermediate when a single catalytic head is bound to the actin filament and that head releases ADP before the free head can rebind to the filament, leading to detachment at saturating ATP. Wang et al. (16) previously showed that detachment of a catalytic head from actin while bound to ADP is extremely slow (0.0003 s\(^{-1}\)), consistent with our model of ADP release before detachment. The similarity between our results and those of Kovács et al. (18), summarized in Table 1, supports the conclusion that ADP release from the trailing head is the rate-limiting step before both forward steps and detachment. Also, the observation that both of these transitions are force-dependent is consistent with the observation by Veigel et al. (15) that ADP release is the force-sensitive transition in smooth muscle myosin II.

Although forward steps and detachment are broadly consistent with the scheme of Kovács et al. (18), we find that backsteps are far more frequent than they would have predicted from their lead head ADP release rates. The simplest model of a backstep is that the lead head detaches and rebinds behind the trailing head. This model satisfies a likely structural constraint for NMIIB processivity, that the myosin heads must bind to neighboring sites along actin (18). We find that backstep rates occur at a rate of 0.77 s\(^{-1}\) with no measurable force dependence, whereas the rate measured by Kovács et al. (18) of ADP release from the lead head under rearward intramolecular strain is 0.023 s\(^{-1}\). This rate is 33-fold slower than our backward stepping rate at 0 pN load. Our interpretation, which satisfies both of these results, is that backward steps occur without ADP release from the lead head. Because we find a near zero distance parameter for backward steps, it appears that only small strains are necessary to detach the weakly bound leading head. To explain the reduced number of observed backsteps at limiting ATP, we propose that the trailing head releases ADP and dwells in a nucleotide-free state. Upon ADP release from the trailing head, an additional movement of the trailing lever arm (56) causes the lead head to bind to actin irreversibly, committing the motor to take a forward step.

The stall force is the force at which forward and backward steps occur at the same rate. We find that NMIIB stalls at a force of ~2.2 pN. As other processive myosins (such as myosin V or myosin VI) approach stall, stepping rates are dramatically slowed by the applied force (30, 48, 49). Thus, myosin V and VI may be poorly suited to set a specific actin filament tension and to respond to tension fluctuations in the cell. Kad et al. (53) referred to the forward and backward stepping of myosin V at high force as a "dynamic stall" process. Here, we find an even more dynamic and therefore more unconventional approach to stall. Because the NMIIB dwell times are less sensitive to force, we find many more back-and-forth mechanical transitions at stall.

![FIGURE 7. A model of NMIIB stepping behavior.](image)
CONCLUSIONS

Processive myosin motors must satisfy a set of rigid kinetic constraints. First, the two myosin heads must bind to actin in overlapping time intervals, because processive runs terminate when both heads detach from actin (57). Second, the two heads must turn over ATP and move in a strictly alternating sequence, to ensure that the heads remain on neighboring binding sites on actin (26). This second constraint suggests that independent heads would make many errors and detach prematurely, even for the short runs we observe here (0.5°, or ~6% success for 4 steps). Combined, these two constraints require that the heads must be gated (communicate allosterically) because independent heads are unlikely to move processively. The most likely form of gating is an intramolecular strain-sensitive event in the ATPase cycle because this would allow the two myosin heads to communicate over long distances. In fact, Kovács et al. (18) have proposed that ADP release is itself strain-sensitive based on prior structural work by Iwamoto et al. (56), and as noted above, our force-sensitive forward stepping rates are broadly consistent with their accelerated ADP release rate from the trailing head. We attempted but were unable to directly observe a motor substep upon ADP release, as seen previously in smooth muscle myosin II and myosin I (55, 57, 58). However, our experimental geometry may not be ideal for detecting such a substep.

Our data support a model in which NMIIB takes multiple processive 5.5-nm steps along an actin protofilament (Fig. 7A). One prediction this model makes is that NMIIB should twist actin filaments. At low force, NMIIB walks primarily forward with few back steps to generate tension in a relaxed filament. Once the actin filament is under tension, NMIIB remains bound to the actin filament while taking forward and backward steps at an equal rate near 2.2 pN load. Stall occurs at this load due to the differing load-dependent processes that lead to forward and backward steps. The motor must move forward by 1.6 nm to take a forward step (or detach) but only requires a backward motion of ~0.3 nm to take a backstep (Fig. 7B). For these backsteps, we believe that the lead head ruptures from the filament, whereas the working stroke of the trailing head is reversed under load (perhaps while ATP hydrolysis products are still bound (59)). This ability to stall while actively cycling may be a necessary cellular adaptation for a motor that must maintain tension. If fluctuations cause the load on NMIIB to change, this myosin will step in the proper direction, forward or backward, until it reestablishes a force of 2.2 pN. Thus, NMIIB is ideally suited to maintain tension in a dynamic and rapidly fluctuating cellular environment.

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