A Low Affinity Ground State Conformation for the Dynein Microtubule Binding Domain

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Dynein interacts with microtubules through a dedicated binding domain that is dynamically controlled to achieve high or low affinity, depending on the state of nucleotide bound in a distant catalytic pocket. The active sites for microtubule binding and ATP hydrolysis communicate via conformational changes transduced through a ~10-nm length antiparallel coiled-coil stalk, which connects the binding domain to the roughly 300-kDa motor core. Recently, an x-ray structure of the murine cytoplasmic dynein microtubule binding domain (MTBD) in a weak affinity conformation was published, containing a covalently constrained β+ registry for the coiled-coil stalk segment (Carter, A. P., Garbarino, J. E., Wilson-Kubalek, E. M., Shipley, W. E., Cho, C., Milligan, R. A., Vale, R. D., and Gibbons, I. R. (2008) Science 322, 1691–1695). We here present an NMR analysis of the isolated MTBD from Dictyostelium discoideum that demonstrates the coiled-coil β+ registry corresponds to the low energy conformation for this functional region of dynein. Addition of sequence encoding roughly half of the coiled-coil stalk proximal to the binding tip results in a decreased affinity of the MTBD for microtubules. In contrast, addition of the complete coiled-coil sequence drives the MTBD to the conformationally unstable, high affinity binding state. These results suggest a thermodynamic coupling between conformational free energy differences in the α and β+ registries of the coiled-coil stalk that acts as a switch between high and low affinity conformations of the MTBD. A balancing of opposing conformations in the stalk and MTBD enables potentially modest long-range interactions arising from ATP binding in the motor core to induce a relaxation of the MTBD into the stable low affinity state.

Dyneins comprise one of the three families of cytoskeleton-based molecular motors that generate force and translocate cargo in eukaryotic cells (2). These motors work by coupling high and low affinity binding to microtubule or actin filaments, with force producing conformational changes driven by an ATP catalytic cycle (3–5). The coordination between these steps is critical for efficient linear movement. Force production occurs after tight binding is achieved, in a manner that both moves cargo forward and facilitates motor repositioning to advance another step. Although ATP hydrolysis and product release provide the thermodynamic driving force for motility, binding to the microtubule or actin filaments also provides feedback to the catalytic pocket and influences the catalytic rate.

An understanding of how substrate affinity is achieved and how it is coupled to nucleotide hydrolysis remains an important problem for all three families of motors (dynein, kinesin, and myosin). For dynein, these issues are particularly complex. In contrast to the close proximity of the substrate-binding domains and catalytic sites in kinesin or myosin-type motors (5), the ATP-sensitive interaction of dynein with microtubules occurs through contacts within a relatively small (~125 residue) globular domain (microtubule binding domain, MTBD), located at the distal end of a 10-nm long α-helical antiparallel coiled-coil stalk (6, 7). This stalk projects off the roughly 3000-residue ring-shaped AAA+ motor domain (Fig. 1). The ATP-catalytic site largely responsible for coupling force production with substrate affinity is located on the side of the ring opposite the site where the stalk emerges (8, 9). These two functionally coordinated domains are separated by a distance up to ~25 nm.

In the absence of nucleotide, dynein binds to microtubules with an affinity near 1 µM (10–13), while ATP binding increases the dissociation constant to above 10 µM. Gibbons et al. (10) fused portions of the MTBD and coiled-coil stalk sequence of the murine cytoplasmic dynein to the Thermus thermophilus seryl-tRNA synthetase, and produced a set of constructs in which the heptad repeat register within the coiled-coil was systematically altered. One coiled-coil registry, designated by default as α, yielded high affinity microtubule binding, whereas half-heptad shifts in either direction (designated β and β) showed significantly reduced microtubule affinity. These results led the authors to propose that a nucleotide-dependent change in microtubule affinity is transduced from the motor to the MTBD through a shift in the helical registry of the stalk. Strong support for this model was obtained for the Dictyostelium discoideum dynein by introducing pairs of cysteines into the coiled-coil sequence and oxidizing these residues to force different coiled registries in an otherwise intact motor (11). The disulfide cross-link pattern predicted to yield the α registry also produced a motor with high microtubule affinity. A 4-res-

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⁵ The abbreviations used are: MTBD, microtubule binding domain; PIPES, 1,4-piperazine diethanesulfonic acid; HSCQ, heteronuclear single quantum coherence; TOCSY, total correlation spectroscopy.
idue shift in the cross-link position to the predicted $\beta^+$ helical registry yielded an ATP-independent low microtubule affinity.

Recently, Carter et al. (1) reported a 2.3-Å resolution x-ray structure of the murine MTBD, in which the coiled-coil stalk was locked into the $\beta^+$ helical registry by fusion onto the seryl-tRNA synthetase. This low affinity conformation of the MTBD in native dynein could be attained by one of two general mechanisms. In the absence of adenine nucleotide, the high affinity state of the MTBD could correspond to a low energy “relaxed” conformation, and binding of ATP to the AAA$^+$ ATPase core of the motor would induce a shift in the coiled-coil registry that distorts the conformation of the MTBD and disrupts its inter-actions with the microtubule. Alternatively, the low microtubule affinity state of the MTBD could represent the energy-relaxed conformation, and release of the $\gamma$-phosphate bound to the AAA$^+$ ATPase core would induce a coil registry shift that generates an unstable, but high affinity MTBD conformation.

These two mechanistic alternatives are potentially distinguishable on the basis of microtubule binding affinity and conformation of the MTBD removed from the dynein motor core. A range of affinities, from 1 to $>20 \mu M$, have been reported from microtubule co-pelleting experiments utilizing MTBD constructs from D. discoideum, Saccharomyces cerevisiae, and murine dyneins containing differing proportions of the stalk sequence attached (1, 10–13). However, with the exception of the x-ray work cited above (1), none of the studies provides tertiary detail regarding the relative orientations of the helical registries. We report here an NMR analysis of the D. discoideum MTBD, including establishment of the alignment and registry of the N- and C-terminal helices. To address the role of the helix registry in modulating the transition between the low and high affinity microtubule binding states, we have further carried out binding analyses for the isolated MTBD, the MTBD attached to the complete coiled-coil stalk segment, and a shortened variant containing sequence for the proximal half of the stalk. Backbone$^{15}$N NMR relaxation measurements were then analyzed for evidence of internal dynamics that could provide insight into the conformational transitions of the MTBD.

**EXPERIMENTAL PROCEDURES**

**Polypeptide Expression**—DNA sequences containing the D. discoideum dynein MTBD were isolated from heavy chain constructs by restriction digestion or PCR, cloned into the pET14b plasmid, and introduced into BL21(DE3)pLysS cells for expression. Escherichia coli cultures were diluted into LB or M9 minimal media, grown at 37°C until the A$_{600}$ reached 0.4–0.6, then chilled on ice to room temperature. Polypeptide expression was induced with isopropyl $\beta$-D-galactopyranoside (0.1 mM final concentration) for 4 h. For samples used in NMR analysis, the M9 medium was prepared using 1.0 g/liter of $^{15}$NH$_4$Cl. For NMR samples requiring $^{13}$C enrichment, 2.0 g/liter of [U-$^{13}$C]glucose was used as carbon source.

Soluble polypeptide was isolated by nickel affinity chromatography (Talon resin), followed by dialysis into 20 mM Tris (pH 8.4), 150 mM NaCl, and 2.5 mM CaCl$_2$. The N-terminal His$_6$ tag was removed by thrombin digestion and subsequent rebinding to Talon. This treatment left an N-terminal 8-residue sequence (GSHMLEDP) preceding the initial proline residue of the MT-3 fragment. A stop codon was inserted immediately after the terminal proline. Samples of the MTBD used for NMR analysis were equilibrated into a buffer containing 150 mM NaCl, 20 mM PIPES-d$_{18}$ (Cambridge Isotopes, Inc.), 0.1 mM EDTA (pH 6.50) with 6% $^2$H$_2$O, and concentrated to 0.5 mM by centrifugal ultrafiltration.

For the microtubule binding and CD experiments, the His$_6$ tag leader sequence of pET14b was not removed from the constructs. In addition to the dynein encoding sequences, the MT-1 and MT-2 constructs, respectively, further contained downstream trailing sequences of 24 (HMLEDPAANKARE-AELAAATAEQ) and 11 residues (GFEIDKLGSGC). These additional residues were accounted for in the CD analysis.

**Microtubule Binding Analysis**—For binding analyses (see Fig. 4, B and C), purified MTBD fragments were equilibrated in an assay buffer (20 mM Tris, pH 7.5, 50 mM KCl, 2 mM MgCl$_2$), mixed with Taxol-stabilized bovine microtubules (5 $\mu M$ final dimer concentration), and incubated at room temperature for 20 min. Following sedimentation at 200,000 $\times$ g for 15 min, pellets were rinsed and then resuspended to the original volume. Equal aliquots of the initial mixture, post-centrifuged supernatant, and microtubule pellet were analyzed by SDS-PAGE. Gels were stained with Coomassie Blue and digitized on a Fujifilm LAS-3000 imager; band intensities were quantified with ImageJ (NIH).

**Saturation and dissociation constants were calculated using a one site-specific binding equation in Prism 5 (GraphPad Software, Inc.).**

**NMR Spectroscopy and Analysis**—NMR resonance assignment and relaxation experiments were recorded at 20°C on a Bruker Avance 600 MHz spectrometer equipped with a triple resonance cryoprobe. Relaxation measurements were also carried out on a Bruker Avance 800 MHz spectrometer equipped with a triple resonance cryoprobe. Experimental data were processed with Felix (Felix NMR Co.) software. The $^{13}$C and $^{15}$N chemical shifts were referenced indirectly using the $\gamma_{13C}/\gamma_{1H}$ and $\gamma_{15N}/\gamma_{1H}$ ratios, respectively (14). Two-dimensional $^1$H-$^1$N HSQC (15), three-dimensional HNCO (16, 17), HNCACB (17, 18), HN(CA)CO (19, 20), and HN(CO)CACB (21) experiments were carried out to establish the backbone resonance assignments. Three-dimensional HBHA(CO)NH (19, 22), HC(C)H-TOCSY, and (H)CH-TOCSY (23) experiments were used to establish a partial assignment of the side chain $^1$H and $^{13}$C resonances as well. Three-dimensional $^1$H-$^1$N-$^1$H NOESY (24) and $^{13}$C-$^1$N-$^1$H NOESY (25, 26) spectra with a 70-ns mixing time were obtained for establishment of $^1$H-$^1$H distance constraints.

 Backbone amide $T_1$ and $T_2$ relaxation measurements (27) were carried out with a recycle delay of 1.6 s. Longitudinal relaxation delays of 0.05, 0.08, 0.13, 0.20, 0.08, 0.30, 0.50, 0.80, 0.30, and 1.35 s were used. Delays of 16.96, 33.92, 50.88, 16.96, 84.80, 118.72, 152.64, 84.8, and 186.56 ms were used to measure transverse relaxation. The exponential decay constants were estimated via Monte Carlo analysis of the uncertainties with the Curvefit program version 1.30 (kindly provided by A. G. Palmer). $^1$H-$^1$N NOE relaxation measurements (27) using a 5.0-s recycle delay were collected in duplicate to provide uncertainty estimates.
Dynein-Microtubule Affinity

Order parameter analysis was carried out with the ModellFree program version 4.20 (28, 29) (kindly provided by A. G. Palmer). An axially symmetric molecular diffusion tensor analysis was conducted using the homology model coordinates derived from the murine MTBD domain x-ray structure (1), as described below. The optimal dynamical model for analysis of each backbone amide was assigned according to the previously described protocol (28).

Circular Dichroism Spectroscopy—A set of four CD spectra of the MT-2 (71:30) construct were measured at 25 °C in a 25 mM phosphate buffer (pH 7). Data were collected over a spectral range of 178 to 260 nm with a path length of 1 mm, a bandwidth of 1 nm, and a 1.0-s response time at a scan rate of 20 nm/min on a Jasco J-720 spectropolarimeter (Jasco, Inc.). The protein concentration of 37.7 μM was determined via quantitative amino acid analysis. Helical content was estimated with the secondary structure analysis programs Selcon3 (30, 31), CDSSSTR (30, 32), and CONTIN (32, 33).

Molecular Modeling of the Coil Registry in the D. discoideum MTBD—Sequence alignment of the murine and D. discoideum MTBD domains indicated a single residue insertion in the D. discoideum sequence. Comparison between the backbone φ and ψ dihedral angles predicted from the TALOS (34) chemical shift analysis of the D. discoideum MTBD and the x-ray structure of the murine MTBD (1) were consistent with the BLASTP prediction of an insertion of methionine at position 73. This alignment and the coordinates for the A molecule in the crystallographic asymmetric unit of the murine MTBD were submitted to the MODELLER 9 version 6 program (35) to generate five models of the D. discoideum MTBD. The models yielding the best molpdf and DOPE (36) values were identified. The three-dimensional 1H–15N–1H NOESY and three-dimensional 13C–15N–1H NOESY spectra were examined to unambiguously assign the backbone resonances of the non-proline residues (BioMagResBank entry 16781), as well as for most of the Hα and Hβ positions. In addition, partial assignments of the aliphatic side chain resonances were obtained from three-dimensional HCCH-TOCSY (23) experiments. The chemical shifts for the 13C and 15N resonances of the protein backbone provide robust predictors of the local conformation. Although the initial analyses of protein backbone chemical shifts were focused on the prediction of secondary structure (37), more detailed structural interpretations have proven feasible. For proteins of less than 100 residues, backbone and 13Cβ chemical shift data are sufficient to reduce the search space for de novo structure prediction methods so as to generate structural models of comparable quality to those obtained by conventional NOE-dependent NMR analysis (38, 39). Rather than intrinsic limitations in the chemical shift data, it is primarily the expanding size of the search space that limits the present utility of backbone chemical shift data for determining the structures of larger proteins (40). Reversing this logic, similarity between the experimental backbone chemical shifts and those predicted for a given structural model of the protein provides a useful fingerprint for the accuracy of that model.

The TALOS program (34) provides robust predictions of the protein backbone torsion angles based on the chemical shifts of the backbone and 13Cβ resonances. In analyzing 200 proteins with both high resolution x-ray structures and essentially complete backbone resonance assignments, the TALOS program confidently predicts the local backbone geometry for between 65 and 74% of the residues with an error rate of 2.5% (in most cases Δφ or Δψ > 60°). Excluding that 2.5% population, the average similarity between the predicted and observed φ and ψ torsion angles for these 200 x-ray structures is ~13° (41).

The most probable (φ,ψ) backbone torsion angles for the residues of the D. discoideum MTBD were predicted by the TALOS program. Confident predictions were obtained for 71% of the 124 residues between the N- and C-terminal prolines. Of these 88 residues, only Lys46, Phe92, and Gly93 had a predicted order parameter analysis of the D. discoideum MTBD is comparable with that observed for the 200 proteins analyzed in the TALOS study (41). The placement of the helical segments in the chemical shift analysis of the D. discoideum MTBD agrees quite closely with those reported in the murine x-ray structure, and a reasonably good agreement exists for the local backbone conformation in most of the interhelical segments (Fig. 2). As further indicated by the 15N relaxation analysis considered below, the differences in backbone conformation for the C-terminal residues arise from the fraying of the truncated C-terminal helix in the isolated D. discoideum MTBD domain. Chemical shift analysis of the linker segment conformation between helix α1 and α2 is limited by the presence of the PKPPTP sequence for residues 25 to 30.

There are two other clusters of residues for which the backbone geometry predicted for the D. discoideum MTBD differed
substantially from the murine x-ray structure, the linker segments between helix $\alpha_2$ and helix $\alpha_3$ and between helix $\alpha_5$ and helix $\alpha_6$. Not only does the “erroneous” confident TALOS prediction for Lys46 lie within the $\alpha_2$-$\alpha_3$ linker segment, there are four other residues of this segment for which TALOS provided only provisional predictions that deviate strongly from the conformation in the murine x-ray structure (Fig. 2). In particular, TALOS provisionally predicted a common right-handed $\alpha$-helical conformation for Gly43, whereas this residue lies in the mirror left-handed $\alpha$-helical conformation in the x-ray structure. As discussed at length in the analysis of the murine x-ray structure (1), the two nonequivalent molecules in the crystallographic asymmetric unit have markedly differing backbone geometries for this $\alpha_2$-$\alpha_3$ linker segment. Although the crystallographic A molecule yields a modestly better root mean square deviation for the NMR-predicted backbone torsion angles throughout the full protein sequence, it should be noted that the crystallographic B molecule provides an appreciably better agreement with the chemical shift predictions for residues Gly44, Lys45, Leu47, and Glu48.

Significant deviations between the chemical shift predictions and the backbone geometry in the x-ray structure were observed for *D. discoideum* residues Gly91, Phe92, and Asp93, which lie within the linker segment between helix $\alpha_5$ and helix $\alpha_6$ (Fig. 2). As discussed below, our $^{15}$N relaxation measurements indicate that Asp93 exhibits the most extensive conformational dynamics observed within the protein backbone, excepting the mobile C terminus.

Helical Registry in the Isolated *D. discoideum* MTBD—Given the 50% identity between the *D. discoideum* and murine amino acid sequences, the level of agreement between predicted and observed local backbone geometries of these homologous MTB domains is consistent with the adoption of very similar tertiary structures. However, in contrast to the present NMR work, the murine dynein fragment used for x-ray analysis was artificially locked into the $\alpha$-$\beta$ registry via fusion of both ends of the coiled-coil onto the seryl-tRNA synthetase (1, 10). In the crystallized construct, three heptad repeats of the coiled-coil are inserted between the synthetase splice site and the strictly conserved proline residues at the stalk-MTBD boundary.
Dynein-Microtubule Affinity

To more clearly understand the structural basis of the conformational communication between the MTBD and the site of ATP hydrolysis in the dynein core, it is critical to establish the nature of the helical registry within the low energy conformational ground state of the unconstrained MTBD. We used the MODELLER program (35) to construct a set of homology models for the D. discoideum MTBD. The model yielding the best molpdf value was then used to predict NOE cross-peaks that indicate the helical registry (Fig. 3, A and B).

Analysis of dynein sequences from various organisms previously predicted the stalk to be composed of an antiparallel coiled-coil extending to the two strictly conserved prolines at either end of the MTBD headpiece (6, 7, 10, 42). However, as revealed by x-ray analysis of the murine MTBD (1), the distal end of the coiled-coil extends past these prolines to form one face of the MTBD itself. The shorter helical segments of the domain (α1 to α6) are packed on top of these N- and C-terminal helices. In particular, helices α2, α4, α5, and α6 all form extensive hydrophobic interactions with the C-terminal helix, whereas the N-terminal helix has a few contacts only with the α4 helix. All of these contacts are located between the two strictly conserved proline residues that mark the boundary between the stalk and the globular binding domain (1, 10, 42).

From the x-ray analysis, it further appears that interactions between the three-turn coiled-coil helices within the proline to proline fragment (green segments in Fig. 3A) are not only important in forming the registries that modulate affinity, but also play a functional role in establishing the overall architecture of the globular domain that binds microtubules.

The asymmetry of these interactions led Carter et al. (1) to propose that the regulatory shift in helical registry occurs via the sliding of the N-terminal helix with respect to the more structurally constrained C-terminal helix. With helical registry constrained by either fusion to the seryl-tRNA synthetase (10) or by disulfide cross-linking between the antiparallel helices (11), the transition between the low affinity β⁺ registry and the high affinity α registry is believed to arise from a 4-residue shift (Fig. 3C).

Protein instability has confounded attempts by us and others (13) to obtain complete side chain assignments and determine a full solution structure of the MTBD. Thus to directly demonstrate that the isolated D. discoideum MTBD exists in the β⁺, as opposed to the α helical registry, we characterized specific NOE interactions that span between the N- and C-terminal helices (Fig. 3B and Table 1). The amide protons in an α-helix are generally too distant from the amide protons in an antiparallel helical coil to give rise to observable NOE cross-peaks. Furthermore, in the D. discoideum MTBD homology model not a single methine or methylene proton within the helical segment from Ile⁸ to Ile¹³ is within 4.5 Å of any amide proton in the C-terminal helix spanning residues Lys¹⁰² to Pro¹²⁶. In fact, there are only two methyl groups within the...
N-terminal helix that have the centroid of their methyl protons within 4.5 Å of an amide proton in the C-terminal helix (Ala6(H6)-Trp110(H9) and Val10(H7)-Trp110(H9)), whereas two additional methyls lie within 5.0 Å of an amide proton in the C-terminal helix (Ala6(H6)-Ala113(HN) and Ile13(H13)-Leu107(HN)). When combined with the NOE interactions of the Ile3(H6) (discussed below), the three-dimensional 1H-15N-1H and 13C-15N-1H NOEY spectra were examined for cross-peaks consistent with these interhelical interactions as well as with the analogous interactions formed by shifting 4 residues with respect to the C-terminal helix, as anticipated for the α registry.

The excellent dispersion within the two-dimensional 1H-15N HSQC spectrum (Fig. 1) was exploited to minimize complications arising from accidental degeneracy in the analysis of these three-dimensional spectra. The 1H-15N HSQC cross-peaks are well resolved for each of the amides in the C-terminal helix that are predicted to yield observable interhelical NOE interactions in the MTBD homology model as well as for the amides that would adopt the equivalent positions in the α registry of the coiled-coil. As a result, ambiguities in the assignment of NOE cross-peaks can only arise regarding the carbon-bound proton. To identify potential accidental degeneracies, all protons within 5.5 Å of each examined amide of the C-terminal helix were predicted from the homology model structure. During the NOE analysis we noted that the amides of both Gin114 and Tyr117 have NOE connectivities corresponding to the upfield shifted resonance for the δ methyl of Ile3 with no alternative assignment available within 5.5 Å in the MODELLER-based homology model yielding the best molpdf value. However, the homology-modeled structure having the best DOPE (36) score predicts a gauche−X1 rotamer for the Ile3 side chain that brings the δ methyl within 5 Å of the amides for both Gin114 and Tyr117. This Ile3 rotamer is also sterically accessible in the molpdf-selected homology model.

As summarized in Table 1, cross-peaks are observed for most of the interactions predicted from the β+ helical registry, whereas none of the NOE connectivities predicted for the α helical registry are observed. In a few instances, other protons within 5.5 Å of each examined amide potentially give rise to cross-peaks with 1H or 13C chemical shifts that overlap with those of the interhelical interactions, although in the case of the Ala6(H6)-Trp110(H9) interaction, the similar shifts for the Ala111 methyl can be discounted because the observed cross-peaks are considerably stronger than that expected for a 5.4-Å interproton separation between Ala111(H6) and Trp110(H9). Despite the modest number of short range amide-side chain interactions predicted between the helices of the coiled-coil, the observed NOE cross-peak pattern is fully consistent with a β+ registry, whereas the analogous predictions for the α registry fail completely.

**Microtubule Binding Affinity of D. discoideum MTBD-Stalk Constructs**—The demonstration that the isolated MTBD adopts the low affinity β+ coiled-coil registry implies that the high affinity α registry, characteristic of the complete motor in the absence of ATP, corresponds to an energetically unfavorable conformational state of the MTBD. To address whether energetics of the coiled-coil registry could modulate conformational states of the MTBD, we carried out parallel binding affinity measurements on a set of D. discoideum constructs containing different amounts of coiled-coil sequence.

As diagramed in Fig. 4A, we expressed and purified variously sized fragments of the dynein heavy chain from E. coli. The largest fragment, MT-1 (115:140; numbering from the two internal conserved prolines as indicated in Ref. 10, Pro3252–3254), comprises the globular contact site and the entire two helical regions defined by the two outermost of the 4 highly conserved proline residues (42). The sequence for the MT-2 fragment (71:30, Asp3296–Leu3520) begins and ends in the mid-

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**TABLE 1**

**Correlation of predicted and observed interhelical NOE cross-peaks for D. discoideum MTBD**

| β+ Registry | α Registry |
|-------------|------------|
| Distance     | 1H        | 1H        | 13C       | 13C       |
| Ile6(H6)     | Gin114(H6) | 4.1 Å      | +         | Ser110(H6) |          |
| Ly6(H6)      |             | 4.7 Å      | +         | Leu23(H6)  | −         |
| Ala6(H6)     | Trp110(H6)  | 3.9 Å      | +         | Gin114(H6) | −         | Ov∗       |
| Ala111(H6)   |             | 4.7 Å      | +         | Trp110(H6) | −         |
| Val10(H7)    | Trp110(H6)  | 4.5 Å      | +         | Gin114(H6) | −         |
| Ile3(H6)     |              | 4.8 Å      | +         | Ov∗        | W         | Ala113(H6) | −         |

*Interproton distance following an unhindered χ1 rotation of the Ile 3 side chain from the trans to the gauche− rotamer.

† Similar chemical shift for the Ala111 methyl at 5.4 Å.

Ov denotes predicted degeneracy, W denotes a weak cross-peak.

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**FIGURE 4. MTBD constructs and microtubule affinity.** A, schematic illustrating the helix-contact domain-helix organization of the dynein stalk/MTBD, and the relative placements of the MT-1, -2, and -3 fragments. B, Coomassie-stained gel panels showing the microtubule affinities of the three fragments at different concentrations. Each gel panel presents the MTBD fragment in the tubulin mixture before centrifugation (M), and the supernatant (S) and pellet (P) pairs after centrifugation. The micromolar amount of the MTBD fragment in M is also indicated at the left of each panel. Roughly equal masses of MTBD fragments are shown in the no tubulin control panel to demonstrate their solubility in the absence of tubulin. Band intensities for near equimolar amounts of MT-1, MT-2, and MT-3 in the presence of the 5 μM tubulin dimer reflect the variation in their molecular weights. C, one site-specific binding plot of MT-3, comparing the amounts pelleted per microtubule with the amount present in the initial mixture. The MT-3 fragment reaches a binding saturation close to an equimolar ratio with tubulin, allowing a reasonable estimation of its dissociation constant. Similar analyses with the other two fragments do not contain a saturation phase and are not included here. See text for details. Error bars indicate the root mean square deviations among three independent experiments run on separate gels.
The preferential binding of MT-3 much more tightly. At low concentrations, nearly all of the contrasts, the full-length coiled-coil construct MT-1 binds tubulin. Higher ordered, radial arrays of a closely related, nearly identical multimers may exist at lower concentrations of MT-1. Although the MT-2 construct contains a larger proportion of the coiled-coil sequence than MT-3 and a smaller proportion from the homology model of the MTBD. Coordinates corresponding to the middle of the coiled-coil stalk might disrupt the interactions at the distant tubulin interface may be provided by a recent analysis by Höök and colleagues (43). They reported CD measurements on a set of MTBD stalk constructs from rat dynein, including the full-length stalk sequence and a series of heptad deletions from both the N-terminal and C-terminal coiled-coil segments. Heptad repeat deletions within the stalk led to a disproportionate decrease in helical content of the polypeptide. The authors concluded that either the MTBD must be conformationally altered or that regions of the coiled-coil must be disorganized. Because the partial stalk construct, MT-2 (71:30), exhibited little affinity for microtubules, we measured the CD spectra over a wavelength range from 178 to 260 nm, to estimate the helical content for this polypeptide (supplemental Fig. S1). Three different secondary structure analysis programs yielded an α-helical estimate of 49–50%, which is appreciably less than either the isolated MTBD or that anticipated for the high affinity MT-1 construct containing the complete stalk sequence. This result is consistent with the conclusions of Höök et al. (43).

**NMR Relaxation Analysis of Backbone Conformational Dynamics in the MTBD**—Currently, little is known about the conformation of the MTBD in its high affinity state. Mutational studies on the *D. discoideum* MTBD have identified several residues that, when modified, strongly affect the microtubule interaction (7). Analysis of the murine x-ray structure indicated that most of these critical residues reside in helices α1 and α3 and lie across the face of the MTBD opposite the attachment to the coiled-coil stalk. Given the relatively modest differences in the free energy of binding between the high and low affinity states implied by microtubule binding studies, the conformational reorganization at the interaction interface could be reasonably small.

We sought to characterize the conformational dynamics of the MTBD through NMR relaxation analysis. 15N T1, T2, and heteronuclear NOE relaxation measurements were performed on the backbone amides of the MTBD. The elongated shape of the MTBD (1, 12) implies that the effective molecular tumbling rate, as sensed by NMR relaxation measurements, will be sensitive to the orientation of each 1H-15N bond vector with respect to the major axis of rotational diffusion. Coordinates from the homology model of the *D. discoideum* MTBD were used to model this asymmetry in rotational diffusion during the analysis of the 15N relaxation data with the ModelFree program (28, 29). An axial diffusion ratio of 1.26 and an effective molecular correlation time of 10.6 ns at 20 °C were derived. These rotational diffusion values were then used to derive order parameters for the individual backbone amide resonances as a function of the optimized motional models (28) (Fig. 5). Except...
for residues within the interhelical segments on either side of the α5 helix and in the flexible C terminus, the mobility of the backbone in the picosecond-nanosecond time frame appears to be significantly restricted, as has commonly been observed for more stable globular proteins. Few 15N relaxation data are available for the interhelical segment between helix α1 and helix α2, due to the PKPPTP sequence of residues 25 to 30.

For relaxation data collected in a 14.1 tesla magnetic field on a 600 MHz spectrometer, the ModelFree analysis tentatively identified 5 residues having small (~1Hz) chemical exchange contributions to the 15N T2 relaxation. However, analogous relaxation measurements made on an 800 MHz spectrometer did not corroborate a magnetic field strength-dependent enhanced line broadening for these residues. As a result, there appears to be no evidence for significant internal motion of the backbone in the slower time regime.

The absence of evidence for either chemical exchange line broadening or substantial dynamics in the picosecond-nanosecond time frame for the residues in the helical segments of the MTBD offers support for a model based on the x-ray structure of the murine MTBD locked in the β+ coiled-coil registry. In an asymmetrically tumbling protein, if the individual amide H-N bond vectors are incorrectly oriented with respect to the axis of the molecular diffusion tensor in the structural model, the resultant errors in the relaxation analysis will manifest themselves as an increase in the apparent internal motion (44).

**DISCUSSION**

The microtubule affinity of the dynein motor is driven by coordinated conformational changes in distinct binding and catalytic domains that are linked by an antiparallel coiled-coil stalk. Results from mutational analyses of key residues in the MTBD (7, 10) and forced alterations in the coiled-coil registries (10, 11) strongly support a dynamic modulation of affinity, effected through sliding of the coiled-coil strands in the stalk. Our data presented here address the mechanism by which the energetic coupling between ATP binding and microtubule affinity is modulated.

Our NMR analysis of the *D. discoideum* MTBD indicates that, in solution, the conformation of the isolated domain is consistent with a structure determined by x-ray analysis of the conformationally constrained murine MTBD (1). Not only are the helical sequences similarly positioned, but also the N- and C-terminal helices of the MTBD interact to adopt the low microtubule affinity, β+ coiled-coil registry. When measured in an independent binding assay, the isolated MTBD also shows a relatively weak affinity for microtubules. Our results therefore indicate that the β+ registry defines the low energy, weak binding conformation of this dynein functional domain.

However, when the MTBD is attached to the full-length stalk segment, in vitro binding assays demonstrate that it switches to a higher microtubule affinity conformation. Previous correlations between affinity and coiled-coil stalk conformations indicate that a shift to the α-registry is required to achieve tight microtubule binding (1, 10, 11). We infer from this work that energetics of coiled-coil interactions within the full stalk must therefore favor the α registry, and these must be sufficient to overcome the weak binding ground state conformation of the MTBD.

Partial truncation of the coiled-coil stalk may act to decrease the relative stability of the α registry vis-à-vis the β+ registry, such that the differential free energy is no longer sufficient to drive the conformation of the MTBD into the high-energy strong binding state. Moreover, interactions within the retained half of the coiled-coil sequence proximal to the MTBD appear to shift the conformational equilibrium sufficiently far away from the β+ coil registry energetic minimum that the microtubule binding interactions of the MTBD become weakened still further. Consistent with this idea, full-length stalks appear to be required for complete folding of the coiled-coil (43), and motor domains containing a partial length stalk move at a reduced rate with elevated ATPase rate (1). These results suggest that the entire stalk is necessary and is optimally tuned for full modulation of microtubule affinity.

The NMR relaxation data, whereas indicating an increased mobility in the linker regions before and after the α5 helix, do not otherwise offer direct evidence for significantly populated alternative conformations. Although the shift in helical registry must surely necessitate some structural rearrangement in the MTBD, the differences in affinity that characterize the tight and weak binding conformations are perhaps achieved through only modest changes along the interaction surface.

We propose here a model in which the dynein MTBD/stalk assembly acts as a counterpoised switch, modulating differential stabilities of the α and β+ registries against weak and tight binding conformations of the MTBD. By destabilizing the α registry relative to the β+ registry, adenine nucleotide binding to the dynein motor would allow the MTBD to relax back to its weak binding conformational ground state. Subsequent nucleotide hydrolysis and product release would then produce motor-domain conformations that favor the α registry, thereby driving the MTBD into the high affinity conformation. EM analyses of a related axonemal dynein revealed that the stalk appears stiffer in the absence of ATP (45). In the high MT affinity state, the coiled-coil would be in its energy optimum α registry, which may account for the observed stiffness. Binding to the microtubule could then alter the strained geometry of the MTBD in such a way that the energetics within the stalk are shifted, ultimately influencing the enzymatic activity in the catalytic pocket of the motor core. Thus despite the large size of the dynein motor, even slight conformational changes propagated around the motor ring could produce positional differences between the AAA-4 and -5 domains that flank the MTBD. These positional differences would only have to be of sufficient magnitude to overcome the strain energies inherent between the α and β+ helical registries to modulate the motor affinity for the microtubules.

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