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ARTICLE

The dynein stalk head, the microtubule binding-domain of dynein: NMR assignment and ligand binding

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Abstract Dynein is a motor ATPase, and the C-terminal two-thirds of its heavy chain form a ring structure. One of protrudings from this ring structure is a stalk whose tip, the dynein stalk head (DSH), is thought to be the microtubule-binding domain. As a first step toward elucidating the functional mechanisms of DSH, we aimed at the NMR structural analysis of an isolated DSH from mouse cytoplasmic dynein. The DSH expressed in bacteria and purified was coprecipitated with microtubules, suggesting its proper folding. Chemical shifts of the DSH were obtained from NMR measurements, and backbone assignment identified 94% of the main-chain N–H signals. Secondary structural prediction programs showed that about 60% of the residues formed α-helices. A region with cationic residues K58 and R61 (and possibly R66 as well), and another with R86, K88, K90, and K91, were found to form α-helices. Both of these regions may be important in the formation of the DSH-binding site to a microtubule that has a low pI with a number of acidic residues. Two synthetic peptides containing the sequence of the α-helix 12 of β-tubulin, considered to be important in binding to DSH, were investigated. Of these two peptides, the one with higher helix-formation propensity appeared to bind to DSH, since it precipitated with DSH in a nearly stoichiometric manner. This suggested that the α-helicity of this region would be important in its binding to DSH.

Keywords Dynein stalk head · Microtubule-binding · NMR analysis · Backbone assignment · Secondary structure

Abbreviations

DSH	Dynein stalk head
AAA	ATPases associated with diverse cellular activities
IPTG	Isopropyl-β-D-thiogalactopyranoside
EGTA	Ethyleneglycol-bis-β-aminoethylether-*N*,*N*,*N*′*N*′-tetraacetic acid
MOPS	3-Morpholinopropanesulfonic acid
DTT	Dithiothreitol
p-ABSF	4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride
DSS	Sodium 2,2-dimethyl-2-silapentane-5-sulfonate
TFE	2,2,2-Trifluoroethanol

Introduction

Dynein, a microtubular motor, is a huge protein complex containing one to three heavy chains of about 500 kDa each, along with some intermediate and light chains (Mitchell 1994; Hirokawa 1998; Vale 2003). Analyses of the amino acid sequences of dynein heavy chains have shown that they belong to the expanded AAA protein family (AAA+) (Neuwald et al. 1999). The overall molecular structure of dynein is not known, but electron microscopic observations have revealed a ring-shaped "head" and two protruding parts: a thick "tail" and a thin "stalk" (Samso et al. 1998; Burgess

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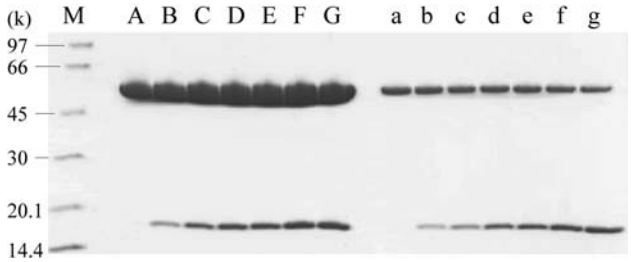

Fig. 1 SDS-PAGE pattern of coprecipitation experiments with microtubules. Lane M indicates molecular markers. The seven lanes on the left (A–G) are for the resuspended pellet, and the seven on the right (a–g) are for the supernatants. While the concentration of microtubules was fixed at 20 μM, that of DS(8:5) was varied: 0 μM (A and a), 2.5 μM (B and b), 5 μM (C and c), 7.5 μM (D and d), 10 μM (E and e), 15 μM (F and f), and 20 μM (G and g). DS(8:5) and microtubules were mixed at the concentrations above and centrifuged to obtain supernatants and pellets (see Materials and methods for details). The upper bands are due to tubulin, and the lower ones are due to DS(8:5)

et al. 2003; for a molecular model sketch of dynein, see Figs. 1 and 4 in Burgess et al. 2003). The head is composed of the C-terminal two-thirds of the heavy chain and consists of six AAA domains, AAA1–AAA6, in tandem. AAA1 is the domain occupying the N-terminus of the ring structure, i.e., the head, and shows ATPase activity, which is responsible for motility. AAA2, AAA3, and AAA4 each have an ATP-binding motif and might also have the ability to hydrolyze ATP (King 2000; Takahashi et al. 2004). Though their functions have not been clarified, AAA2–AAA4 might regulate dynein function in relation to ATP concentration (Kon et al. 2004). AAA5 and AAA6 have no ATP-binding motifs, and their roles other than maintaining the structure are not known.

The stalk is a protrusion between AAA4 and AAA5; it is composed of an antiparallel coiled-coil, 10–15 μm in length, and a globular domain on the tip called the dynein stalk head (DSH) consisting of about 120 amino acid residues (Gee et al. 1997). DSH is thought to bind to and dissociate from microtubules in a reversible manner, depending on the ATP hydrolytic steps of the catalytic cycle. However, DSH is far from the catalytic site of AAA1 in the head. This has raised the question of how the ring structure transmits the enzymatic reaction information to the DSH. Gibbons et al. (2005) investigated various chimeric proteins containing DSH and hypothesized that the coiled-coil part would form different registries of hydrophobic residues during the catalytic cycle. This would cause a conformational change in DSH to control its microtubule-binding affinity. However, the molecular structure of DSH has not been revealed and the hypothesis has not been confirmed.

Structural analyses of DSH would provide clues to this reversible process of binding to and dissociating from the microtubules and to the coupling between the association state and the enzymatic reaction. For the first step, we aimed to analyze the structure of free DSH. We made a construct, "DS(8:5)", consisting of 136 residues, which correspond to E3278–E3413 of the mouse cytoplasmic dynein. Various NMR measurements were carried out, and 94% of the main-chain signals of N–H were identified by backbone assignment. Based on these chemical shifts, secondary structural features were obtained by structural prediction software. In addition, two chemically synthesized peptides, each containing the sequence of the α-helix 12 of β-tubulin, which is considered important in DSH-binding, were separately added to DS(8:5), and only the one with the higher potential to form the α-helix was found to bind to DS(8:5).

Materials and methods

Peptide synthesis

Peptides were chemically synthesized and purified as described previously (Shimizu et al. 2005). Based on the sequence corresponding to α-helix 12 of mouse β-tubulin, two peptides were synthesized. One peptide, named "B12st", was designed to have this very sequence (FTEAESN MNDLVSE YQ, 16 amino acid residues). The other, "B12TN", was made to have two of this sequence in a tandem manner with a linker of five amino acid residues between them to maintain α-helical registry (EFTEAES NMNDLVS EYQSLLS EFTEAES NMNDLVS EYQ, 38 amino acid residues).

Construction of the expression system

DNA coding DS(8:5), corresponding to E3278–E3413 of the mouse cytoplasmic dynein, was amplified by PCR from cDNA (NCBI accession number AY004877). A forward primer including an *Nde*I site (5′-GGG CAT ATG GAG GAC CTG GAT AAA GTG-3′) and a reverse primer with an *Eco*RI site and with a His_6-tag at the C-terminus of DS(8:5) (5′-GGG GAA TTC TTA ATG ATG ATG ATG ATG ATG CTC ATT CCT CAG GGG CTC-3′) were used for the amplification. The PCR product was subcloned into pET-17b (Novagen, Madison, WI, USA).

Expression and purification of the sample

Escherichia coli BL21-CodonPlus (DE3) cells (Stratagene, La Jolla, CA, USA) harboring pET17b/DS(8:5) were cultured at 37°C for 5–6 h in 1 l of LB or CHL medium (Chlorella Industry, Tokyo, Japan). ^{15}N-labeled or $^{13}C^{15}N$-labeled CHL medium was used for producing isotope-labeled DS(8:5). The expression was induced by 0.4 mM IPTG, and the bacteria were further cultured at 22–24°C for 2–3 h. The bacteria were then harvested at 4°C and stored in a freezer.

The protein preparation was done on ice or at 4°C. The bacterial pellet was thawed and resuspended in 20 ml of sonic buffer (10 mM Na-phosphate (pH 8.0), 250 mM NaCl, and 20 mM imidazole) with a small amount of DNase I and lysozyme, and then was ultrasonicated. The homogenate was centrifuged at 18,000 rpm for 20 min to obtain the supernatant.

Three milliliters of Ni-NTA gel (Qiagen, Valencia, CA, USA) equilibrated with sonic buffer was added to the supernatant. The mixture was stirred gently for 30 min and poured into a column. After the column was washed with the sonic buffer, the product was eluted with a solution containing 10 mM Na-phosphate (pH 8.0), 250 mM NaCl, and 300 mM imidazole. The fractions with large quantities of protein were collected (about 3 ml) and dialyzed against the dialysis buffer (20 mM MOPS-NaOH (pH 7.0) and 100 mM NaCl). This protein solution was passed through a 1.5 ml Q-sepharose (GE Healthcare, Uppsala, Sweden) column equilibrated with the dialysis buffer. The flow-through fractions (about 4 ml) were applied to a 120 ml Sephacryl S-300 column (GE Healthcare) equilibrated with size-exclusion buffer (for coprecipitation experiments, 20 mM $NaHCO_3$ (pH 8.0) and 100 mM NaCl; for NMR measurements, 10 mM Na-phosphate (pH 6.7) and 80 mM NaCl). Three-milliliter fractions were collected, and the monomeric DS(8:5) with high purity was obtained.

Preparation of NMR samples

The DS(8:5) purified as described above was concentrated by ultrafiltration and added to certain reagents; the final composition of the solution was 0.13 mM DS(8:5), 10 mM Na-phosphate (pH 6.7), 75 mM NaCl, 10% D_2O, 0.02% sodium azide, 1 mM DTT, 0.1 mM *p*-ABSF (a protease inhibitor), and 5% sucrose (for possible protein stabilization). A BMS-005 V NMR tube (Shigemi, Tokyo, Japan) was used after triple siliconization with L-25 (Fuji Systems, Tokyo, Japan), unless otherwise stated. The NMR tube containing DS(8:5) was centrifuged manually and degassed by aspiration.

NMR measurements

NMR measurements were performed using a Unity INOVA 600 MHz spectrometer (Varian, Palo Alto, CA, USA) equipped with a Cold Probe (Varian) at 20°C. The measurement duration was varied from 1 h to 1 day depending on the S/N ratio for ^{1}H–^{15}N HSQC and about 1 week for the three-dimensional measurements. As the internal standard for ^{1}H chemical shifts, 2.5 mM DSS was used, and all the signals measured were calibrated accordingly. The chemical shifts of C^{α} and C^{β} were observed by using the pulse sequences HNCACB, CBCA(CO)NH, C(CO)NH, HNCA, and HN(CO)CA. The data showing good signal separation and S/N ratios were averaged. Those of H^{α} were observed by HC(CO)NH and ^{15}N edited TOCSY, and the data were averaged, while those of C=O were observed by HNCO. All NMR experiments were performed using a Varian BioPack module on the operation program VNMR. The NMR data were processed by the program NMRPipe (Delaglio et al. 1995) and analyzed by the program SPARKY 3 (Goddard and Kneller 1999).

CD measurements

The CD spectrum was recorded on a J-720 spectropolarimeter (Jasco, Tokyo, Japan). The light pass length was 1 mm, and the measurement temperature was 25°C.

HPLC analysis

Proteins and peptides in solution or dissolved precipitate were analyzed with reverse-phase HPLC using YMC-Protein-RP (C4) columns (YMC, Kyoto, Japan).

Coprecipitation with microtubules

Tubulin was isolated and purified from porcine brains (Murphy and Borisy 1975) and stored at −80°C. For polymerization, appropriate amounts were thawed and equimolar paclitaxel (Sigma, St. Louis, MO, USA) was added to them, and incubated at room temperature for 10 min. The suspension was centrifuged through a cushion of 5% sucrose in a coprecipitation buffer (100 mM MOPS-NaOH (pH 7.0), 2 mM $MgCl_2$, and 0.2 mM EGTA) at 16,000 rpm for 10 min. The pellet was resuspended with the coprecipitation buffer including small amounts of paclitaxel.

DS(8:5) at concentrations ranging from 0 to 20 μM was added to 20 μM of microtubules in 100 μl of coprecipitation buffer. After incubation at room temperature for 30 min, the mixtures were centrifuged at 16,000 rpm for 10 min. This centrifugal force was sufficient for pelleting most of the microtubules, but small fractions of tubulin, perhaps small fragmental microtubules, remained in the supernatant. This should not affect the results of the coprecipitation experiments. The pellets were resuspended in 100 μl of the coprecipitation buffer and analyzed by SDS-PAGE along with the supernatants.

Results and discussion

Coprecipitation with microtubules

Since DSH is considered the ATP-dependent microtubule-binding region of dynein (Mizuno et al. 2004), DS(8:5),

our DSH sample, was expected to possess binding affinity to microtubules. To verify this, coprecipitation experiments were carried out. When 2.5 μM of DS(8:5) was mixed with 20 μM of microtubules, about half or more of the total DS(8:5) was found in the pellet (Fig. 1). It should be noted that DS(8:5) alone did not precipitate upon centrifugation. Together with other data, the binding constant would be on the order of 5–10 μM. This should agree with the report of chimeric proteins by Gibbons et al. (2005), who described the binding constant of 2.2 μM as a "tight binding" state and that of 12.1 μM as a "weak binding" state. Thus, DS(8:5) was proven to have significant microtubule-binding affinity, indicating that the protein was properly folded.

Optimal conditions for NMR measurements

For good triple resonance spectra with sufficient S/N ratios, samples with high protein concentrations should be prepared. However, when DS(8:5) was concentrated to 0.16 mM or more, the solution became opaque with NMR signal broadening, probably due to sample aggregation. We tried various physiological solution conditions, but the results were similar to one another. The measurements of DS(8:5) were, therefore, carried out at about 0.13 mM to avoid aggregation. Since this concentration was relatively low for three-dimensional NMR measurements, more scans were needed, which led to a prolonged measurement.

We then checked the time-dependent quality change of the sample in a triply siliconized NMR tube using ^{1}H–^{15}N HSQC spectra, which showed that the dispersion and S/N ratio of the spectra of 0.13 mM DS(8:5) were good even after 2 weeks. On the other hand, the stability of DS(8:5) in a glass tube without any treatment, or in a Teflon or polypropylene tube, was much worse.

The NMR measurements were, thereafter, performed using triply siliconized tubes. Nonetheless, new signals grew with time even when these tubes were used. These signals should be attributed to protein deterioration, and were distinguished from the originally observed signals in the assignment.

Backbone assignment

As described above, DS(8:5) at a concentration of 0.13 mM in the triply siliconized glass tubes seemed to maintain its native structure for 2 weeks. Under this condition, backbone assignments were carried out using the chemical shifts of C^{α} and C^{β} from various measurements.

Our sample had 136 amino acid residues in addition to the starting methionine and the His_6-tag. We identified 128 of 136 C^{α}'s, 123 of 134 C^{β}'s (excluding 2 glycine residues), and 121 of 129 N–H's (excluding 7 proline residues). Based on the assignments of the N–H signals, 122 of 136 C=O's and 46 of 138 H^{α}'s (including 2 additional ones from glycine residues) were identified. The chemical shift data have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu/) under BMRB number 11013. Complete assignments were not possible due to signal broadening, overlapping, or weakness. A representative NMR spectrum, the ^{1}H–^{15}N HSQC spectrum with assignments, is shown in Fig. 2 for the N–H signals.

Some of the signals, considered to be derived from the deterioration of the sample (see the section above), were assigned separately. They were revealed to correspond to

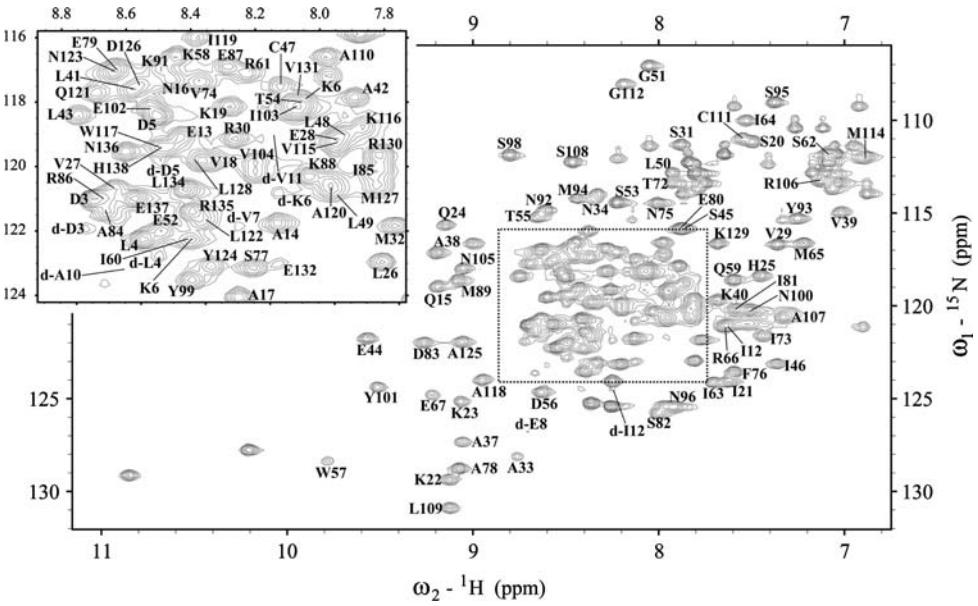

Fig. 2 ^{1}H–^{15}N HSQC spectrum of DS(8:5) with backbone assignment. The amino acid residues are denoted by single-letter abbreviations and their residue numbers. Some of the signals newly appeared and grew over time, which were considered to be attributed to sample deterioration. They were assigned separately and were revealed to correspond to the residues D3-E8 and A10-I12, designated such as d-D3 and d-I12

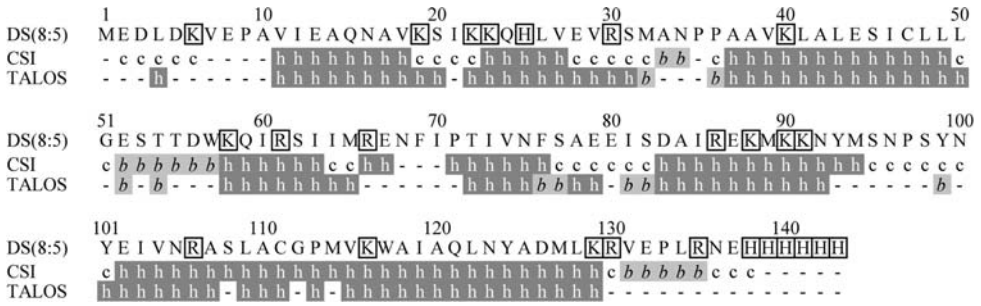

Fig. 3 Secondary structure predictions of DS(8:5). Single letters after DS(8:5) indicate amino acid residues, while basic residues are framed, and the numbers above them indicate their positions from the N-terminus. The "h" letters on a dark background indicate residues predicted to be α-helices, and the italic "b" letters on a light gray background indicate those predicted to be β-structures. The "c" letters indicate random coil structures and the "-" symbols mean that no particular secondary structures were predicted at these regions. The programs CSI and TALOS predicted that 55.9% and 59.4% of the amino acid residues should form α-helices and 9.1% and 6.3% of that should form β-structures, respectively

the residues E2-I12, i.e., N-terminal residues of DS(8:5), denoted on the ^{1}H–^{15}N HSQC spectrum (Fig. 2) such as d-D3 and d-I12. Thus, this sample appeared to lose its native conformation from its N-terminal region.

Prediction of the secondary structure

The secondary structure of DS(8:5) was predicted by the programs CSI (Wishart and Sykes 1994) and TALOS (Cornilescu et al. 1999) based on the chemical shifts. The results are shown in Fig. 3.

The CSI and TALOS results agreed with each other to a considerable extent. In particular, both programs predicted that V11–V18, K23–V27, A37–L49, K58–I63, T72–N75, D83–N92, E102–A107, L109–C111, P113, and V115–K129 were α-helices, and that E52 and T54 were β-structures. On the other hand, some minor differences were found between the two predictions. For example, CSI predicted that the region E52–W57 would form a continuous β-structure and another, E102–K129, would form a continuous α-helix, whereas TALOS predicted there were a few residues without any particular secondary structures. A couple more examples are described later.

Several N-terminal and C-terminal residues are thought to be part of an antiparallel α-helical coiled-coil (Gee et al. 1997). However, the results from both prediction programs indicated that the coiled-coil structure would not be formed; the secondary structure at the N-terminus should be a random coil, while that at the C-terminus was predicted to be a β-structure by CSI, whereas TALOS did not predict the presence of any particular secondary structures. The probable antiparallel coiled-coil regions at both termini were presumably not long enough to form stable α-helical structures because of the truncation. Since the deterioration of the sample is thought to occur from its N-terminus (see the section above), unfolding of the antiparallel coiled-coil would be one of the causes of the instability of DS(8:5). It would be of interest to introduce mutations to stabilize the coiled-coil and to prevent the deterioration; this might make a complete structural determination possible.

CSI and TALOS predicted that respectively 55.9% and 59.4% of the amino acid residues should form α-helices. These predictions agree with the data of molar ellipticity, −18,300 at 222 nm by the CD measurements, from which the α-helical content was estimated to be about 60% (Chen et al. 1974).

Sequence alignments and possible microtubule-binding regions

The sequence alignments of four cytoplasmic dyneins from different species along with DS(8:5) are shown in Fig. 4. Tubulin has a low pI. In particular, the α-helix 12 of β-tubulin has several aspartic acid and glutamic acid residues, and it has been reported that mutations of some of these acidic residues weakened the binding of the kinesin motor domain (Uchimura et al. 2006). Since dynein and kinesin are thought to bind to overlapping regions of tubulin in microtubules (Mizuno et al. 2004), the acidic residues of the α-helix 12 of β-tubulin should be important for the binding of dynein. At the microtubule-binding site of DSH, therefore, there should be basic residues for binding to the α-helix 12. With DS(8:5), cationic residues cluster in a region with K58, R61, and R66, and in another with R86, K88, K90, and K91. Homologous clusters are also found in different dyneins. The programs for the secondary structure predicted these regions to be α-helices. These basic residues should be exposed to solvent, and these α-helices should constitute the microtubule binding region (it should be noted that CSI predicts R66 to be α-helical, whereas TALOS predicts that there will be no particular secondary structures). An experiment with mutant dynein recombinants also revealed that at least the former region was important for microtubule binding (Koonce and Tikhonenko 2000).

Fig. 4 A sequence alignment of DSHs. The sequence of DS(8:5) in this study (except for the starting methionine and final His_6-tag) and the corresponding regions of cytoplasmic dyneins from other species, derived by the BLAST program on the NCBI homepage (http://www.ncbi.nlm.nih.gov/BLAST/), are aligned. The identities to DS(8:5) of the dyneins from *Danio rerio* (zebrafish), *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Tetrahymena thermophila* are 84.6%, 75.7%, 57.4%, and 33.8%, respectively. Framed letters indicate basic residues, while shaded letters indicate acidic residues. The symbol "-" means that there is a gap in the amino acid sequence in comparison with other dyneins

DS(8:5) has several basic residues in a cluster: K19, K22, K23, and H25, in addition to those described above, and it is reported that this region may also be important for binding to microtubules (Koonce and Tikhonenko 2000). However, the cytoplasmic dynein from *Tetrahymena* has R3185 as the only basic residue in the corresponding region. This region may contribute to the binding either because of these basic residues or just by maintaining the structure.

Interaction between DS(8:5) and synthetic peptides derived from the α-helix 12 of β-tubulin

Since the α-helix 12 of β-tubulin is considered important for DSH-binding (see the section above), peptides containing this sequence may interact with DS(8:5). The sequence of the α-helix 12 of β-tubulin showed remarkable conservation; the one from mouse differed from that from yeast by only one amino acid: at the second position from the N-terminus, there is a serine residue for yeast and a threonine residue for mouse. In this study, two peptides were chemically synthesized; the one named B12st (16 amino acid residues) was designed to have the very sequence of α-helix 12 of the mouse β-tubulin, while the other, B12TN (38 amino acid residues) was designed to have two of this sequence in a tandem manner (see Materials and methods). B12TN was considered to have a higher helix-forming propensity than B12st; the sequence should be long enough for possible formation of an α-helical structure, whereas B12st might not be.

B12st or B12TN was added to ^{15}N-labeled DS(8:5) at a 10:1 molar ratio, and the ^{1}H–^{15}N HSQC spectrum was measured. Whereas no obvious effects were observed with B12st, with B12TN the S/N ratio was worsened by about 30%, though signal patterns were not changed. It should be mentioned that, at the same time, a small amount of precipitate was formed. Reverse-phase HPLC analysis confirmed that the precipitate consisted of DS(8:5) and B12TN at a molar ratio of 1:1.8, which would be a 1:0.9 molar ratio of DS(8:5) and B12TN dimer, perhaps α-helical coiled-coil. It was, therefore, supposed that the precipitate was formed by stoichiometric binding of DS(8:5) and α-helical coiled-coil dimer of B12TN. The signal patterns remained unchanged, indicating that only unbound DS(8:5) showed NMR signals and that the precipitated one did not. The reduction of unbound DS(8:5) would be the reason for the decrease in the S/N ratio.

Whereas we did not detect any interaction between B12st and DS(8:5), B12TN was likely to bind to DS(8:5), as shown above. B12TN was so designed to have higher potential to form an α-helix than B12st that B12TN might form a certain structure possibly like that in tubulin itself, to interact with DS(8:5). We performed CD measurements of these peptides with TFE at concentrations from 0% to 60% (Fig. 5). Without TFE, both peptides showed CD spectra of typical random coils. It was revealed, however, that B12TN exhibited a higher degree of α-helix-formation under the influence of TFE judged from the CD intensities at 222 nm. Curve-fitting calculations were performed with an assumption of a two-state transition model (Morii et al. 1997), and free energy changes for α-helix-formation in water (i.e., without TFE) were obtained: 14.0 ± 3.5 kJ/mol for B12st or 6.0 ± 1.3 kJ/mol for B12TN. This suggests that B12TN has a higher α-helical propensity as compared

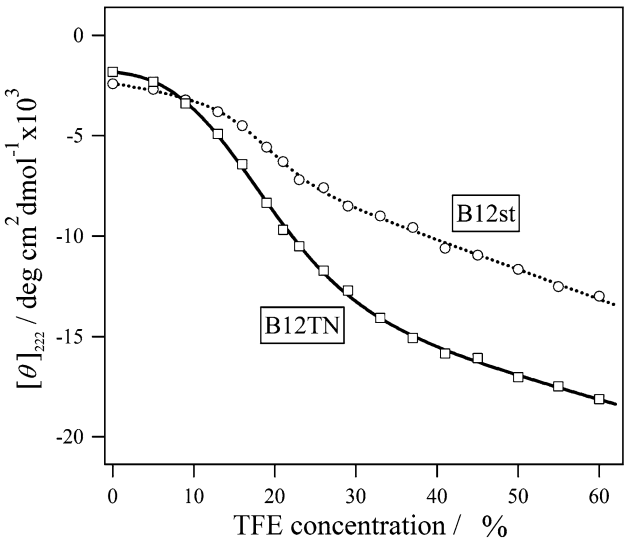

Fig. 5 Molar ellipticities at 222 nm of synthetic peptides with various concentrations of TFE. CD spectra of synthetic peptides were measured at peptidyl residue concentrations (designated as "Mr", a multiple of the peptide concentration and the number of residues) of 2 mMr, corresponding to the molar concentration of 0.13 mM for B12st and 0.053 mM for B12TN. The data were collected in a solution of 10 mM Na-phosphate (pH 8), 75 mM NaCl, and various concentrations of TFE from 0% to 60%; TFE facilitates formations of secondary structures such as α-helices (Buck 1998). Circles and squares indicate the values obtained for B12st and B12TN, respectively, and the lines represent the best-fitting curves after the equation described (Morii et al. 1997)

to B12st under similar conditions. Thus, formation of an α-helical coiled-coil dimer of B12TN might be triggered by the interaction with DS(8:5) even in an aqueous solution without TFE. The reason why the precipitation took place is currently unclear.

We also investigated whether or not microtubules might affect the ^{1}H–^{15}N HSQC spectrum of ^{15}N-labeled DS(8:5) at a molar ratio as high as 0.8:1 (tubulin:DS(8:5)). While signal patterns were unchanged, the S/N ratio decreased significantly as the concentration of microtubules was increased. DS(8:5) bound to microtubules would not show NMR signals because of its much higher molecular mass. Since only unbound DS(8:5) would give the signals, it seemed likely that reduction of free DS(8:5) caused the decrement of the S/N ratio.

These results would be in good agreement with the hypothesis that the α-helix 12 of β-tubulin should be important for DSH-binding, and they suggest that this sequence's α-helix-formation should be a key for the binding. While details of the precipitation are under investigation, the synthesis of new peptides that should bind to DSH but should not cause precipitation may give clues not only for the determination of the binding residues by NMR measurements but also for the binding mechanism of DSH to microtubules.

Conclusions

We constructed an expression system for a protein corresponding to the dynein stalk head (DSH), called DS(8:5), from mouse cytoplasmic dynein, and then expressed and purified this protein. Coprecipitation experiments with microtubules confirmed that DS(8:5) possessed a binding affinity to microtubules, and its dissociation constant was on the order of 5–10 μM. The results are in good agreement with a previous report (Gibbons et al. 2005), suggesting that the protein is properly folded.

Although it was not possible to perform a complete structural analysis of DS(8:5), we obtained valid NMR signals so that 94% of the main-chain signals were assigned. Secondary structural predictions obtained by CSI and TALOS agreed well with each other. The content of α-helices was about 60% according to either program, consistent with the estimation from the CD spectrum.

Basic residues are considered important in binding to microtubules. There are a few clusters of cationic residues along the amino acid sequence of DS(8:5): one in the region with K58, R61, and R66, and another in the region with R86, K88, K90, and K91. Sequence alignment revealed that cytoplasmic dyneins from other species also have clusters of basic residues in these regions. The analysis of chemical shift data predicted that both regions of DS(8:5) were α-helical. Another region, containing K19, K22, K23, and H25, might contribute to the binding affinity.

Judging by changes in the NMR signals, DS(8:5) should deteriorate from its N-terminus. In addition, secondary structural prediction programs showed that both N-terminal and C-terminal regions were unlikely to form an antiparallel coiled-coil. The instability of the sample should be attributable to the non-rigid structural nature of both termini. The formation of a correct antiparallel coiled-coil, either by changing the lengths of both termini or by introducing mutations, would improve the stability of the sample for the complete structural analysis.

The α-helix 12 of β-tubulin is considered important for the binding of DSH and microtubules. We chemically synthesized two peptides containing the sequences of the α-helix 12. The one with a higher helix propensity formed precipitation with DS(8:5) in an almost stoichiometric manner. This may be in good agreement with the hypothesis that the α-helix 12 of β-tubulin would be important for the binding. New peptide synthesis is under way to identify the binding residues of DSH and to reveal the DSH-microtubule binding mechanism.

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