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Dimerization of the Highly Conserved Light Chain Shared by Dynein and Myosin V*

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The M_r 8,000 light chain originally identified in *Chlamydomonas* flagellar dynein is also a component of both cytoplasmic dynein and myosin V. Furthermore, this small protein has been implicated as an inhibitor of neuronal nitric oxide synthase, suggesting that it may play multiple regulatory roles within the cell. Covalent cross-linking of both dynein and myosin V using 1,5-difluoro-2,4-dinitrobenzene revealed that this light chain exists as a dimer *in situ*. This observation was confirmed using two additional amine-selective cross-linking reagents (dimethyl pimelimidate and disuccinimidyl suberate). When expressed as a C-terminal fusion with maltose-binding protein, the presence of the light chain caused the recombinant molecule to dimerize. Analysis of fusions containing truncated light chains identified the predicted amphiphilic helix (residues 14–32) as sufficient to cause dimerization; cross-linking required a second helical segment (residues 33–46). Together the data presented suggest that two light chains interact to form a parallel dimeric structure. This arrangement has significant implications for the potential functions of this highly conserved molecule and suggests a mechanism by which it might dissociate nitric oxide synthase.

Dyneins are highly complex molecular motors that translocate the cargo to which they are attached along microtubules (see Refs. 1 and 2 for review). Molecular analysis of components from both cytoplasmic and flagellar outer arm dyneins has revealed that these microtubule motors share a M_r 8,000 polypeptide (actual mass = 10.3 kDa) that has been very highly conserved throughout evolution (~90% sequence identity between the *Chlamydomonas* and human proteins) (3, 4). In *Drosophila*, partial loss-of-function mutations in this protein lead to morphogenetic defects in bristle and wing development, female sterility, and disruption of sensory axon trajectories; total loss induces apoptosis and causes embryonic lethality (5, 6). Mutation of the homologous protein in *Aspergillus* results in a nuclear migration phenotype similar to that observed with dynein heavy chain null mutants (7).

Intriguingly, fractionation of whole mammalian brain extracts revealed at least three biochemically distinct pools of this protein: (i) non-microtubule-associated; (ii) microtubule-associated, eluted with ATP or salt; and (iii) microtubule-associated, not salt-extractable (4). Only the second pool was cytoplasmic dynein-associated, and indeed, the predominant form of this molecule (pool (i)) did not cosediment with microtubules. Likewise, this polypeptide is associated with at least two distinct components of the *Chlamydomonas* flagellar axoneme. There are 8–10 copies of this protein within the outer dynein arm (8); this fraction is absent in axonemes derived from mutants that lack this structure (9), and it may be extracted from wild-type axonemes with 0.6 M NaCl. The second flagellar fraction, which accounts for ~50% of the total axonemal M_r 8,000 LC,[1] is tightly integrated into the axonemal superstructure, is present in outer armless mutants, and is not released by salt treatment.[2]

Recent biochemical analysis of the unconventional actin-based motor myosin V from chick brain identified four distinct LCs as stoichiometric components of that complex (10, 11). One LC of 10 kDa is apparently identical to the M_r 8,000 dynein LC and is present at a stoichiometry of two LCs per myosin V particle (11). Thus both actin- and microtubule-based molecular motors share a common subunit. This observation has raised the possibility that the M_r 8,000 LC is involved in targeting or regulatory events common to these distinct motor enzymes (11).

Considering the multiple pools of this highly conserved LC that are present in whole brain extracts and in flagellar axonemes, it is possible that this protein is employed by a wide variety of cellular systems in a manner analogous to calmodulin. This latter hypothesis has recently received a boost from the observation that the M_r 8,000 LC interacts with the neuronal form of nitric oxide synthase (nNOS) in a yeast two-hybrid system (12). Furthermore, expression of the LC in transfected cells caused down-regulation of nNOS activity; *in vitro* this occurred through a LC-mediated conversion of the active nNOS dimer to a monomeric inactive form.

Considering the variety and importance of the systems in which the M_r 8,000 LC has been implicated and the differences in stoichiometry observed between flagellar outer arm dynein and cytoplasmic dynein/myosin V, it has become of some interest to determine the solution behavior and intermolecular associations of this molecule as they may well provide important clues as to potential function(s). In this report, we demonstrate that the M_r 8,000 LC is a homodimeric protein within both dynein and myosin V, and the data presented define both the interaction domain and orientation of the dimer. The results narrow considerably the possible roles for this molecule in dynein/myosin V function and suggest a mechanism by which

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[1] The abbreviations used are: LC, light chain; HC, heavy chain; IC, intermediate chain; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; DMP, dimethyl pimelimidate; DSS, disuccinimidyl suberate; MBP, maltose-binding protein; nNOS, neuronal nitric oxide synthase.

[2] S. E. Benashski, A. Harrison, and S. M. King, unpublished observations.

this protein might interact with nNOS to dissociate that enzyme complex and thus affect enzymatic activity.

EXPERIMENTAL PROCEDURES

Constructions—The in-frame fusion of the M_r 8,000 LC (U19490) with maltose-binding protein in the pMal-c2 vector (New England Biolabs, Beverly, MA) was described in King and Patel-King (3). For this study, various smaller portions of the molecule lacking discrete predicted secondary structural elements were obtained using the polymerase chain reaction and subcloned across the *Xmn*I and *Xba*I sites of pMal-c2. All constructs were sequenced prior to protein production.

The LC coding sequence also was subcloned into the pET-16b vector (Novagen, Madison, WI) across the *Nde*I and *Bam*HI sites. This resulted in the C-terminal fusion of the LC to a His_{10} tag that is separated from the LC by a factor Xa cleavage site.

Protein Purification—Flagella were obtained from *Chlamydomonas reinhardtii* strain 1132D(−) and demembranated with Nonidet P-40 as described previously (13, 14). Outer arm dynein was extracted with 0.6 M NaCl and purified by sedimentation in a 5–20% sucrose density gradient (15). Purified rat brain cytoplasmic dynein and chicken brain myosin V were the generous gifts of Drs. Kevin Pfister (University of Virginia Health Science Center) and Foued Espindola (Yale University), respectively.

The recombinant LC expressed as a C-terminal fusion with maltose-binding protein was purified by amylose affinity chromatography as described previously (3). For some experiments, the LC was separated from the fusion partner by digestion with factor Xa, which cleaves immediately prior to the first Met residue within the LC.

The His_{10}-tagged LC was purified by affinity chromatography on a chelated Ni^{2+} column. The 1 M imidazole buffer used for elution of the protein from the column was removed by dialysis prior to use.

Covalent Cross-linking—Flagellar axonemes and sucrose gradient-purified outer arm dynein were prepared in 10 mM HEPES, pH 7.5, 5 mM $MgSO_4$, 0.5 mM EDTA, 25 mM KCl, and treated for 60 min with 0–50 mM 1,5-difluoro-2,4-dinitrobenzene (DFDNB; Pierce). The cross-linking reagent was dissolved in methanol, and all samples (including controls) contained a final methanol concentration of 10% (v/v). Cross-linking with disuccinimidyl suberate (DSS; Pierce) was performed under identical conditions as for DFDNB, except that the solvent was dimethyl formamide. For reaction with dimethyl pimelimidate (DMP; Pierce), samples were exchanged into 100 mM triethanolamine, pH 8.2. All reactions were terminated by the addition of an equal volume of 250 mM Tris·Cl, pH 6.8. Cross-linking of fusion proteins, cytoplasmic dynein, and myosin V was performed in a similar manner.

SDS-Gel Electrophoresis and Protein Blotting—Denatured proteins were separated by electrophoresis in 5–15% acrylamide gradient SDS-containing gels as described previously (15). Gels were either stained with Coomassie Blue or were blotted to nitrocellulose in 10 mM $NaHCO_3$, 3 mM Na_2CO_3, 0.01% SDS, 20% methanol. Nitrocellulose blots were probed with affinity-purified antibody R4058 that specifically reacts with the M_r 8,000 LC (3, 4). Antibody reactivity was assessed following incubation with a peroxidase-conjugated secondary antibody using a chemiluminescent detection system (ECL; Amersham Corp.).

Native Gel Electrophoresis—The solution molecular weights of the fusion proteins were determined by native gel electrophoresis using the method of Hedrick and Smith (16). Samples were electrophoresed in a series of gels of different acrylamide concentration and the retardation coefficients (K_R) derived from the negative slope of 100 ($\log(R_F \cdot 100)$) *versus* gel percentage. Molecular mass standards used were jack bean urease (545,000-Da hexamer and 272,000-Da trimer), bovine serum albumin (132,000-Da dimer, 66,000-Da monomer), ovalbumin (45,000 Da), bovine carbonic anhydrase (29,000 Da), and α-lactalbumin (14,200 Da). A plot of $\log K_R$ *versus* $\log M_r$ for these proteins yielded a standard curve from which the molecular weight of the unknowns could be determined directly.

Circular Dichroism Spectroscopy—The concentration of the purified His_{10}-tagged LC was determined from the absorbance at 280 nm using extinction coefficients of 1,280 and 5,690 liters·mol^{-1}·cm^{-1} for Tyr and Trp residues, respectively (17). The CD spectrum of the sample was recorded between 190 and 300 nm using a Jasco J-715 spectropolarimeter. The mean residue ellipticity at 222 nm ($[\theta]_{222}$) was determined, and the approximate α helical content was calculated based on a value of $[\theta]_{222}$ = 30,000 degrees·cm^2·dmol^{-1} for a completely helical molecule.

RESULTS

To investigate the protein-protein associations in which the M_r 8,000 LC is involved within dynein, we tested a series of

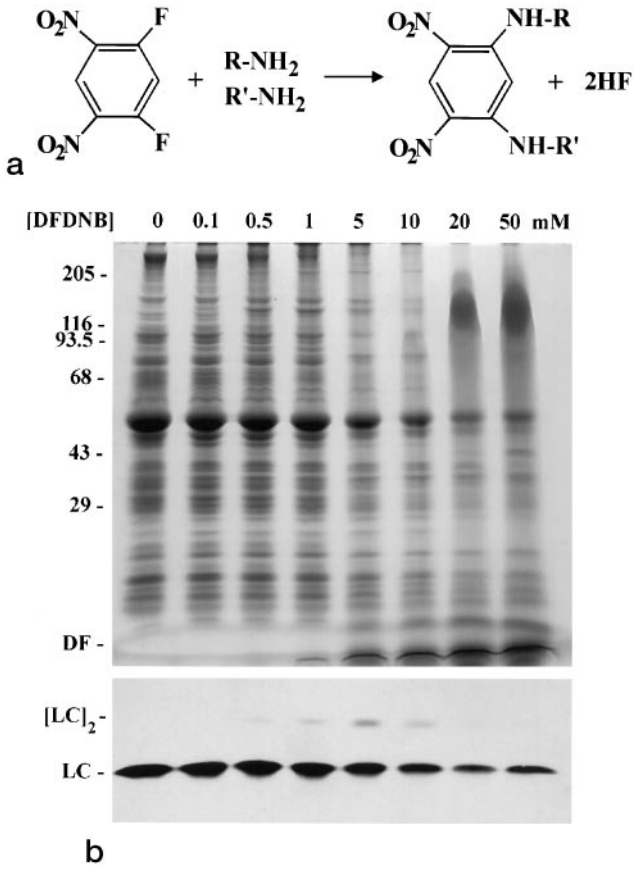

FIG. 1. **Cross-linking of *Chlamydomonas* axonemes with DFDNB.** *a*, scheme describing the reaction of DFDNB with two proteins (*R* and *R′*) containing primary amines. The small size of this reagent limits covalent cross-linking to systems in which the physical separation of the two amino groups is ~3 Å or less. *b*, approximately 150 μg of axonemes were treated with 0–50 mM DFDNB for 60 min. The reactions were quenched by the addition of 0.5 M Tris·Cl, pH 6.8, and following denaturation were electrophoresed in 5–15% acrylamide gradient gels. Gels were either stained with Coomassie Blue (*upper panel*) or blotted to nitrocellulose and probed with the R4058 antibody (*lower panel*) to reveal the M_r 8,000 protein (*LC*). Samples treated with 0.5–10 mM DFDNB yielded one additional immunoreactive band (*$[LC]_2$*) migrating at M_r ~20,000.

cross-linking reagents for their ability to covalently attach the LC to other dynein/axonemal components. Cross-linked products were detected immunologically using a previously characterized highly specific polyclonal antibody (3, 4). Initially, the amine-reactive homobifunctional aryl halide DFDNB was found to yield discrete cross-linked products containing this LC (see Fig. 1*a* for a generalized reaction scheme). This reagent spans a distance of only 3 Å and thus can only cross-link residues that are in close proximity to each other.

Treatment of *Chlamydomonas* axonemes with 0.1–50 mM DFDNB resulted in considerable *intra*axonemal cross-linking as evidenced by the gradual disappearance of discrete protein bands and the appearance of very high molecular weight material that did not enter the gel (Fig. 1*b*, *upper panel*). Immunological analysis of these samples with the R4058 antibody identified a single additional band of M_r ~20,000 that contained the M_r 8,000 LC (Fig. 1*b*, *lower panel*). Two additional amine-selective cross-linking reagents that rely on different chemistries were then used to confirm this result (Fig. 2). Treatment of axonemes with both DMP and DSS resulted in formation of the M_r ~20,000 product. Note that the yields obtained with these reagents, which can span approximately three times the distance of DFDNB, were significantly enhanced over that obtained with DFDNB. Presumably, this re-

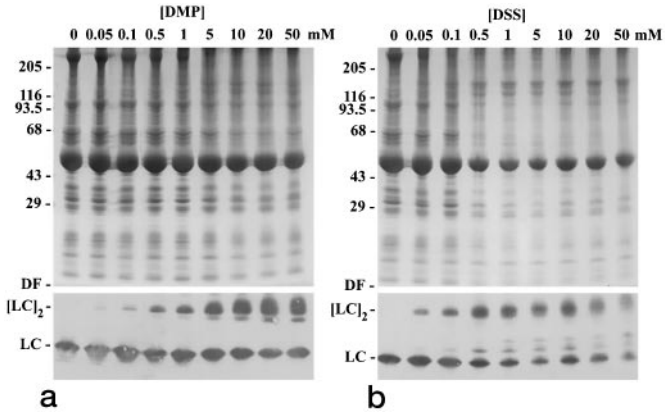

FIG. 2. **DMP and DSS-mediated cross-linking of axonemes.** Two hundred μg of axonemes were treated for 60 min with 0–50 mM DMP (*a*) and DSS (*b*). Samples were electrophoresed in 5–15% acrylamide gels and stained with Coomassie Blue (*upper panels*), or blotted to nitrocellulose and probed with the purified R4058 antibody (*lower panels*). Both reagents generated considerable amounts of an R4058-reactive band migrating at M_r 20,000. The additional minor bands may derive from non-cross-linked protein/reagent adducts that have altered charge due to reduction in the number of primary amines.

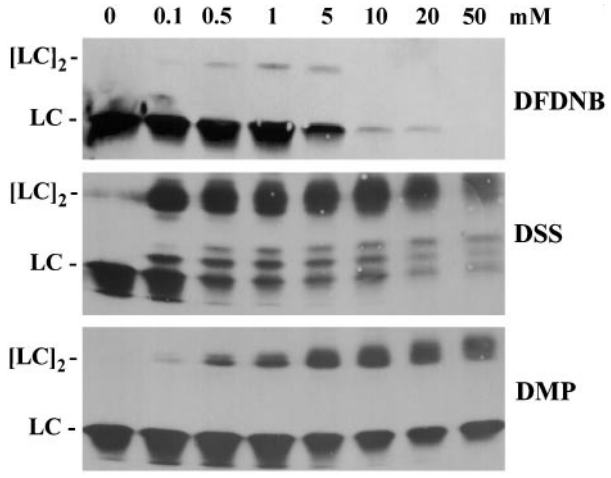

FIG. 3. **Cross-linking of purified outer arm dynein.** Sucrose gradient-purified outer arm dynein (7–10 μg) was treated with 0–50 mM of the cross-linking reagents DFDNB, DMP, and DSS for 60 min. Reactions were quenched with 0.5 M Tris·Cl, pH 6.8. The samples were electrophoresed in 5–15% acrylamide gradient gels, blotted to nitrocellulose, and probed with the R4058 antibody. An immunoreactive band of M_r ~20,000 is evident in dynein samples treated with each of the three different amine-selective reagents.

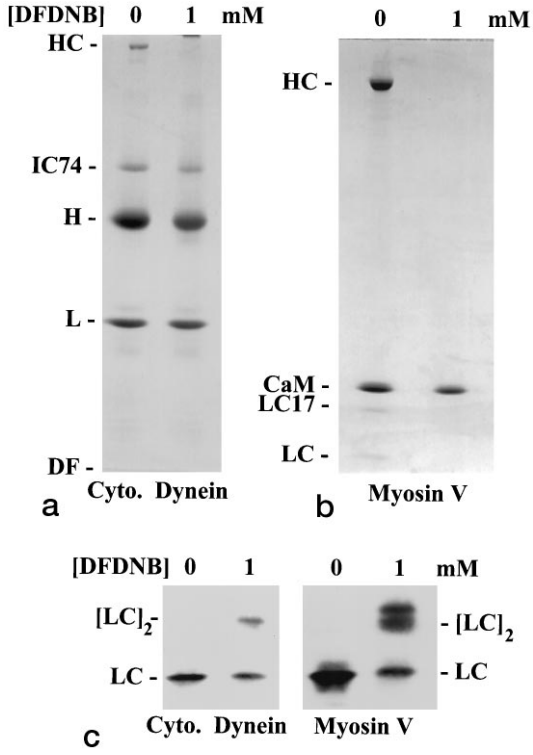

FIG. 4. **DFDNB cross-linking of cytoplasmic dynein and myosin V.** Cytoplasmic dynein immunoprecipitated from a rat brain extract with monoclonal antibody 74-1 (*a*) and purified chick brain myosin V (60 μg) (*b*) were treated with 0 and 1 mM DFDNB for 60 min. Samples were electrophoresed in a 5–15% acrylamide gradient gel and stained with Coomassie Blue. The locations of the various dynein and myosin V components are indicated. In *c*, samples of cytoplasmic dynein and chick brain myosin V were treated with DFDNB, electrophoresed, and blotted to nitrocellulose. The blots were probed with the purified R4058 antibody. Both samples revealed a M_r ~20,000 band following treatment with 1 mM DFDNB. In the myosin V sample, an additional band migrating at M_r ~23,000 also is evident.

flects either less severe orientation constraints or enhanced reactivity of the reagents. With DSS, and to a lesser extent with DMP, minor amounts of products migrating between the M_r 8,000 and 20,000 bands were obtained. The origin of these minor bands remains uncertain, although they may well represent uncross-linked protein/reagent adducts of altered charge.

To determine whether the M_r ~20,000 band represents the cross-linking of the M_r 8,000 LC to another dynein component or to some other axonemal structure, cross-linking reactions were performed using sucrose gradient-purified outer arm dynein (Fig. 3). The M_r ~20,000 band was again observed in DFDNB-, DMP-, and DSS-treated dynein samples, indicating that it indeed derives from an *intra*dynein cross-linking event. The small apparent M_r of this band made it very unlikely to contain other dynein LCs and suggested that the product derived from cross-linking between two of the 8–10 copies of the M_r 8,000 LC that are present within the outer arm dynein. This hypothesis is further supported by the high yields of cross-linked product obtained with DMP and DSS; no other dynein component is present within the purified particle at a stoichiometry sufficient to account for the amount of product generated. With DMP, two electrophoretic variants of the dimeric product were clearly evident (Fig. 3, *lower panel*). Increasing the cross-linker concentration resulted in the conversion of the faster migrating species to a slower form. This observation supports the idea that protein/reagent adducts are being formed that exhibit altered electrophoretic mobility due to the loss of primary amines through reaction with the reagent.

To further analyze the hypothesis that the M_r 8,000 protein exists as a dimer *in situ*, purified samples of cytoplasmic dynein and myosin V, both of which are known to contain two copies of the LC per particle (4, 11) but which share no other common components, were treated with DFDNB. Reaction of cytoplasmic dynein with 1 mM DFDNB resulted in the complete conversion of the HC to a larger aggregate that did not enter the gel (Fig. 4*a*). In contrast, the amount of IC74 present was little affected. Immunological analysis of DFDNB-treated cytoplasmic dynein with the R4058 antibody again revealed a single additional band migrating at M_r ~20,000 (Fig. 4*c*, *left panel*). With myosin V, DFDNB cross-linking resulted in loss of the HC but not calmodulin (Fig. 4*b*), and in the generation of both the M_r ~20,000 band and a second band of M_r ~23,000 (Fig. 4*c*, *right panel*). The former band comigrated with the M_r ~20,000 band from cytoplasmic dynein; the origin of the latter is unclear.

To determine whether the M_r 8,000 LC can indeed dimerize,

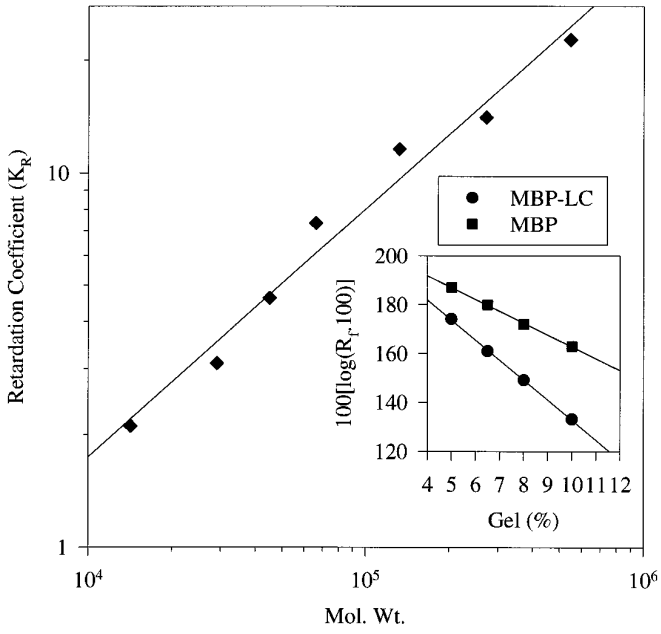

FIG. 5. **Native molecular weight of the MBP-LC fusion protein.** Urease (hexamer and trimer), bovine serum albumin (monomer and dimer), ovalbumin, carbonic anhydrase, and α-lactalbumin were electrophoresed in 5, 6.5, 8, and 10% nondenaturing polyacrylamide gels, and the retardation coefficients (K_R) were calculated from the individual R_F values and plotted *versus* molecular weight to yield a standard curve. The *inset* shows the behavior of MBP and MBP-LC in these gel systems. MBP migrates at 44,160 Da, whereas MBP-LC has a native molecular mass of 94,400 Da.

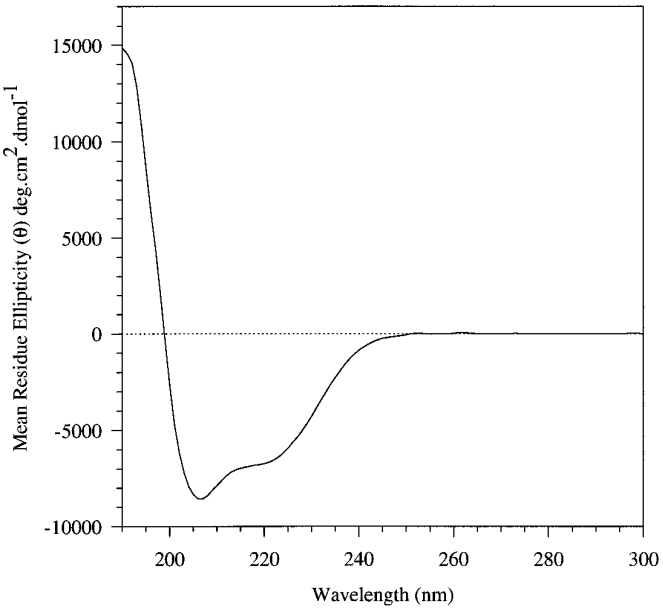

FIG. 6. **Circular dichroism spectroscopy of the purified M_r 8,000 LC.** The LC was prepared at a concentration of 25.6 μM, and the CD spectrum was measured in the far ultraviolet between 190 and 300 nm using a Jasco J-715 spectropolarimeter. The spectrum shows the negative peaks at 222 and 208 nm that are characteristic of α helical structure. From the mean residue ellipticity [θ] at 222 nm, the LC (without the His_{10} tag) is calculated to contain ~30% α helix.

it was expressed *in vitro* as a C-terminal fusion with maltose-binding protein (MBP). In this construction, the LC is separated from MBP by a short (~16 residues) hydrophilic linker that terminates in a factor Xa cleavage site (IEGR). The native molecular weight of both MBP-LC and the MBP-linker moiety obtained following factor Xa digestion were determined using the method of Hedrick and Smith (16) (Fig. 5). The calculated mass of the MBP-linker protein is 42,469 Da, and this molecule migrated in native gels with an apparent mass of 44,150 Da. Therefore, the native MBP-linker protein is monomeric. Addition of the 10.3-kDa LC to MBP yields a fusion protein with a calculated mass of 52,773 Da. This protein migrated with a native molecular mass of 94,400 Da, strongly suggesting that the fusion protein is dimeric in solution. Furthermore, treatment of the native MBP-LC fusion protein with 0.5 mM DFDNB resulted in significant *inter*molecular covalent cross-linking such that ~50% of the fusion protein migrated at M_r ~100,000 following denaturation; MBP alone remained monomeric under the same conditions (not shown). These data indicate that the LC does indeed dimerize *in vitro* and that this interaction may be stabilized by treatment with DFDNB.

Previous secondary structure analysis (3) identified two segments of the molecule that have a very high probability (>90%) of being α helical (residues 14–32 and 34–46); the former section is amphiphilic and therefore is a candidate to mediate protein-protein interactions (18). In addition, that analysis also identified a region (residues 80–88) near the C terminus that had a lower probability (~50%) of being helical. Thus, the M_r 8,000 LC was predicted to contain ~30–45% α helix. To assess the α helical content of the M_r 8,000 LC, the His_{10}-tagged molecule was purified, and the CD spectrum between 190 and 300 nm was recorded (Fig. 6). The spectrum clearly shows significant α helical content as evidenced by the negative peaks at 208 and 222 nm (see Brahms and Brahms (19) for reference spectra). From $[\theta]_{222}$ measurements the molecule is calculated to contain approximately 30% helix after adjustment for the contribution of the His_{10} tag to the random coil component. This analysis provides support for the previous prediction that the region encompassing residues 14–46 is indeed helical.

To determine whether all or part of this helical region represents the dimerization interface and to assess which part(s) of the molecule were involved in the DFDNB-mediated cross-linking reaction, a series of truncated LCs were prepared as C-terminal fusions with MBP (Fig. 7). These truncations were designed so as to remove discrete elements of predicted secondary structure (3). The native molecular weight of each fusion protein was assessed as described above, and each protein also was treated with DFDNB to determine whether it was competent to undergo covalent cross-linking. The properties of the various fusion proteins are tabulated in Table I.

The LCΔ4 and LCΔ5 fusion proteins migrated with native molecular masses close to 90 kDa, indicating that both contain a functional dimerization domain. As these molecules share only the region from residues 1 to 33, the interaction domain must be located within this section. Only the LCΔ4 protein yielded a DFDNB-cross-linked product, suggesting that the second helical segment (residues 33–46) was required for this reaction to occur.

To assess if dimerization was due solely to the amphiphilic helical domain or whether the N-terminal 13 residues also plays a role in LC-LC interactions, an additional fusion protein (LCΔ6) containing LC residues 14–46 was constructed. Approximately 50% of this protein migrated as a dimer in native gels (see Table I); the remainder appeared monomeric. Furthermore, this protein yielded a complex of M_r ~100,000 when treated with DFDNB, indicating that cross-linking did not involve the N-terminal 13 residues; the dimerization helix (residues 14–32) itself does not contain any Lys or Arg residues and thus cannot be involved directly in the cross-linking reaction. These data demonstrate that the LCΔ6 protein contains both the dimerization and cross-linking sites and further indicate that the N terminus is not essential for either property.

Native molecular weight estimates could not be obtained for

constructs LCΔ1-LCΔ3. These proteins share the C-terminal domain (residues 46–91) and appeared to form large aggregates in solution. DFDNB cross-linking yielded significant product only for LCΔ1, presumably because this protein is the only one that has the potential to interact via the dimerization domain and thus bring together the cross-linking sites (residues 33–46) with the correct orientation.

Combined, the properties of the truncated LC fusion proteins indicate that the amphiphilic helical region between residues 14 and 32 is sufficient to cause dimerization of the protein to which it is attached; this domain appears to be at least partly stabilized by the N-terminal residues 1–13. Furthermore, the data are completely consistent with the hypothesis that DFDNB cross-linking occurs solely between amines located in the second helical segment (residues 33 and 46).

DISCUSSION

The M_r 8,000 LC first identified within the *Chlamydomonas* outer dynein arm (3) is one of the most highly conserved proteins known (92% identity between the nematode and human proteins). This molecule is an integral stoichiometric component of both flagellar outer arm and brain cytoplasmic dyneins (4, 8) and also of the actin-based motor, myosin V (11). In addition, this small protein has been implicated in the regulation of nNOS activity (12) and furthermore, is clearly present in several other biochemically distinct compartments where its associations are as yet unknown (4).[2] In this report we have provided evidence to indicate that this polypeptide exists as a dimeric structure, both within dynein and myosin V, and also is of itself sufficient to cause dimerization of a recombinant fusion protein.

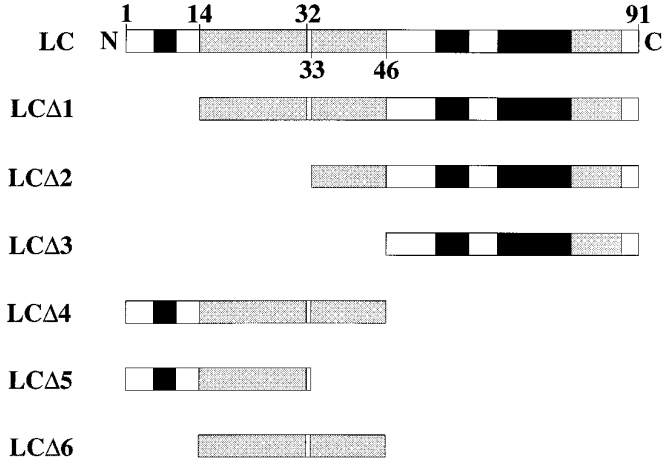

FIG. 7. **Removal of predicted secondary structural elements from the LC.** Secondary structural predictions (3) made using PHD (32) identified several potential elements within the M_r 8,000 LC. Regions predicted to adopt a helical conformation are *shaded*; those expected to be extended sheets are in *black*. The first helical segment (residues 14–33) is amphiphilic with charged/polar and nonpolar residues clustered to opposite sides (3, 20). The six deletion constructs indicated were expressed as C-terminal fusions with MBP to map the sites of dimerization and DFDNB cross-linking.

Substructural Organization of the LC and Orientation of the Dimer—As depicted in Fig. 8, there are five potential ways in which two LCs might interact to form a dimeric structure. Residues 14–33 are all that is required for formation of a dimeric protein. Thus, models that do not involve this region (possibilities *c*–*e* in Fig. 8) must be incorrect. For LCs interacting via the amphiphilic helix, there are two possible arrangements (*a* and *b* in Fig. 8) that derive from the parallel and antiparallel orientations of the LCs. In an attempt to differentiate between these two possibilities, we examined the DFDNB-mediated cross-linking of the MBP/LC fusion proteins. The data obtained indicate that the cross-linking site is located within the second helical segment between residues 33 and 46; this region contains four Lys residues (Lys^{33}, Lys^{38}, Lys^{45}, and Lys^{46}) that could provide the required primary amines. No other regions of the LC were consistently required for cross-linking, suggesting that the cross-link occurs between the same region within the two LCs. The amphiphilic dimerization helix (which is not cross-linked by DFDNB and has no Lys or Arg residues) contains ~20 residues, which is equivalent to a length of ~3 nm (an α helix has a rise of ~1.5 Å per residue) (20), whereas the cross-linking reagent DFDNB can span a maximum distance of only ~3 Å. Thus, it is unlikely that the LCs could become cross-linked by DFDNB if arranged in an antiparallel fashion as the cross-linking sites would be separated by a distance ~10-fold greater than the size of the cross-linking reagent itself. Therefore, it is most likely that the M_r 8,000 LC forms parallel dimers (see Fig. 8*b*). As the other cross-linkers employed in this study (DMP and DSS) have linker lengths of 9.2 and 11.4 Å, respectively, the high yields obtained with these reagents also support a parallel arrangement for the LC dimer.

Structural Implications for the Dimerization Interface—Residues 14–32 of the LC are predicted to form an amphiphilic helix (3). Molecular modeling of this helix (not shown) reveals that one face contains a series of charged residues and is hydrophilic, whereas the other exposes a continuous stripe of hydrophobic side chains that proceed with a right-handed twist about the helix when viewed from the N to the C terminus. Docking simulations for a parallel dimer formed using this region predict that the helix must adopt a right-handed supercoil for LC-LC hydrophobic interactions within this region to account for dimerization.

Implications for Molecular Motors—Both detergent-induced dissociation and cross-linking studies of *Chlamydomonas* outer arm dynein have suggested that the M_r 8,000 LC is located within the IC·LC complex and is therefore to be found at the base of the soluble dynein particle (21, 22). This prediction is

TABLE I
Properties of MBP fusion proteins containing truncated LCs

MBP-LC construct	LC residues present	Calculated molecular weight of fusion protein	Native molecular weight of fusion protein	DFDNB cross-link	Oligomeric state
		M_r	M_r		
LC	1–91	52,773	94,400	Yes	Dimer
LCΔ1	14–91	51,545	n.k.[a]	Yes	n.k.
LCΔ2	33–91	49,506	n.k.[a]	No	n.k.
LCΔ3	46–91	47,955	n.k.[a]	No	n.k.
LCΔ4	1–46	47,450	90,150	Yes	Dimer
LCΔ5	1–33	45,899	87,100	No	Dimer
LCΔ6	14–46	46,072	83,200[b]	Yes	Dimer
MBP-linker	0	42,469	44,150	No	Monomer

[a] The LCΔ1-LCΔ3 fusion proteins appeared to form high molecular weight structures under native conditions. n.k., not known.
[b] A second population of the LCΔ6 protein which migrated as a monomer was also evident in nondenaturing gels.

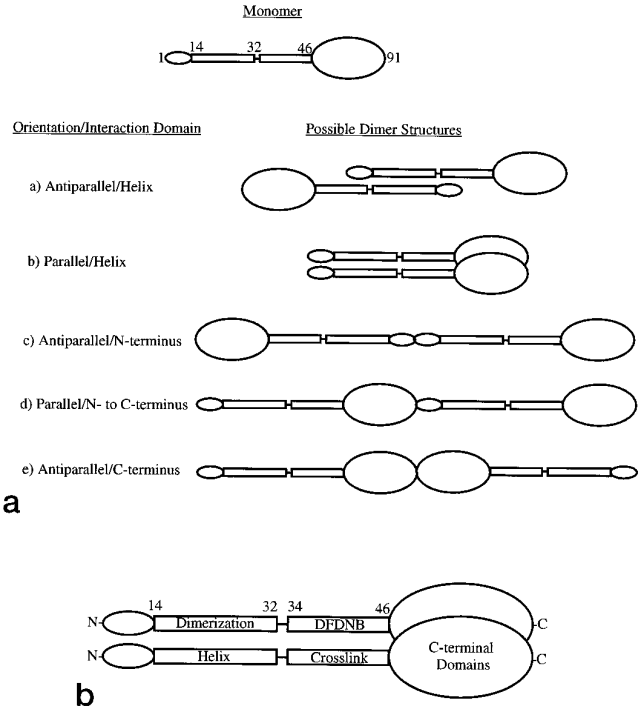

FIG. 8. **Structural orientation of the M_r 8,000 LC dimer.** *a*, a diagram of the M_r 8,000 LC monomer indicating the N- and C-terminal domains and the two adjacent helical regions is shown at the *top*. *Below* are shown the five possible orientations of the LC for dimer formation and the interaction domain(s) that would be responsible for each. *b*, the data obtained from truncated LC fusion proteins are consistent with the LC forming a parallel dimer with residues 14–32 forming the dimerization interface and DFDNB cross-linking requiring residues 33–46. The N-terminal segment likely contributes to the stabilization of the dimerization helix.

supported by studies of dyneins from sea urchin and trout where the IC·LC complex may be isolated by low ionic strength dialyis and sucrose gradient centrifugation (23, 24). In these systems, the M_r 8,000 LC is separated from the HCs and migrates with the ICs at ~7 S in sucrose gradients. Although the stoichiometry of these components has not been reported, it is clear from examination of published gels that the smallest LC[3] is present at a significantly higher stoichiometry than the other LCs within the same particle (for examples, see Refs. 25 and 26). Thus, the presence of a large number of M_r 8,000 LCs appears to be a general feature of outer arm dynein design. This is in clear contrast to the brain motors cytoplasmic dynein and myosin V, both of which contain 2 copies of the LC per motor particle (4, 11).

In cytoplasmic dynein, the LC is probably associated with the ICs at the base of the particle as it is in flagellar dynein, although there is as yet no direct evidence for this assertion. Proteolytic fractionation of myosin V (11) has revealed that the two M_r 8,000 LCs associate with the C-terminal 80-kDa tail domain of that enzyme. Although the M_r 8,000 protein is very highly conserved, sequence analysis reveals no overt similarities between the ICs of cytoplasmic and flagellar dynein and the myosin V tail domain. Thus, it remains unclear how the same LC dimer structure interacts with these disparate molecules. The parallel orientation of the two LCs within the dimer proposed here suggests several features. First, given the symmetric nature of the LC dimer, it likely binds between two common components, *e.g.* the two IC74 proteins in cytoplasmic dynein and the two HCs in myosin V. Second, the regions of the LC that interact with the other components of these motors are probably located within the C-terminal domain of the LC as essentially the entire N-terminal section of this small protein is required for efficient dimerization.

The presence of 8–10 copies of the LC within *Chlamydomonas* outer arm dynein also raises intriguing possibilities. First, there may exist 4–5 dimer binding sites within the two distinct ICs (IC78 and IC69) of that dynein. However, the N-terminal domains of the *Chlamydomonas* ICs show almost no relationship; functionally, this region of IC78 interacts with α-tubulin, whereas the analogous region of IC69 does not (27). The C-terminal domains of these ICs contain the related 5–6 copies of the WD (or G_β)-repeat (28, 29), which are likely to be involved in the formation of a β-propeller-type structure (30). Although it remains possible that the M_r 8,000 LCs interact with individual blades of the β-propeller, the presence of a WD-repeat structure within IC74 of cytoplasmic dynein (29, 31) does not allow this scenario to readily explain the observed difference in LC stoichiometry between the two dynein classes. An alternative possibility is that, within the flagellum, the LC dimers further associate to form higher order oligomers. This possibility is supported by our observation of several discrete multimeric forms of the MBP-LC protein following DFDNB cross-linking. In this scenario, the ICs of outer arm dynein would resemble those of cytoplasmic dynein in requiring only a single dimer-binding interface. This suggestion implies that additional M_r 8,000 LC binding site(s) exist within the axoneme. Indeed, such additional sites must be present, as ~50% of the total M_r 8,000 LC cannot be removed from the *Chlamydomonas* axoneme under the standard extraction conditions that solubilize essentially all of the outer arm. Furthermore, similar amounts of the M_r 8,000 LC remain in axonemes obtained from mutants that completely lack (and never assembled) the outer arm itself. It will clearly be of considerable interest to identify the other axonemal component(s) with which this LC associates.

Interaction of the LC with nNOS—A recent report (12) identified the M_r 8,000 LC as interacting with nNOS in a yeast two-hybrid system and also in transfected mammalian cells. Further *in vitro* analysis revealed that the LC interacted with the nNOS dimer and caused that complex to dissociate into the inactive monomeric form. Thus, disruption of the nNOS complex by the LC led directly to inhibition of nNOS activity. This is potentially of great significance as NO levels are directly modulated *in vivo* by the regulation of NOS activity at the site of synthesis. Secondary structure predictions (made using PHD) (32) (not shown) for the region of nNOS (residues 163–245) which interacts with the LC (12) identified an amphiphilic segment that could contribute to dimerization of nNOS. This suggests that the M_r 8,000 LC may simply exert its effects on nNOS through binding of the amphiphilic dimerization helix of the LC to the similar domain within nNOS. This hypothesis predicts that the inactive form of nNOS consists of a single synthase protein with one copy of the M_r 8,000 LC bound via LC residues 14–32.

In conclusion, we demonstrate here that the highly conserved M_r 8,000 LC found in dynein and myosin V exists as a parallel homodimeric structure. The data provide clues as to the role this molecule may play within these motor systems and suggest a potential mechanism to explain the down-regulation of nNOS activity by the LC. Further detailed structural/functional studies will undoubtedly lead to additional insight into the mechanism of action of this intriguing protein.

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[3] The smallest LC within outer arm dynein from sperm of the sea urchin *Strongylocentrotus purpuratus* is specifically recognized by an antibody (R4058) made against the *Chlamydomonas* M_r 8,000 LC (S. M. King and A. G. Moss, unpublished observations).

dynein, myosin V, and sea urchin dynein, respectively. We are also grateful to Drs. Greg Mullen, Walfrido Antuch, and Zheng-yu Peng for assistance with CD spectroscopy and molecular modeling, and Jed Podoloff and Susan Young for assistance with some experiments.

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Dimerization of the Highly Conserved Light Chain Shared by Dynein and Myosin V

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