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A Circular Dichroic Study of Helical Structure in Flagellar Dynein†

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ABSTRACT: The circular dichroic spectra of outer arm dynein from sea urchin sperm flagella, of its separated α and β heavy-chain complexes, and of the two major fragments produced by tryptic digestion of the β heavy chain have been measured over the range 190–240 nm. Although the spectra show significant individuality, in all cases they qualitatively resemble those of typical globular proteins with mixed regions of α-helix and β-sheet (α/β-type structure) or with separate α-helix- and β-sheet-rich regions ($\alpha+\beta$-type structure). Quantitative analyses of the spectra by both constrained and unconstrained least-squares curve-fitting procedures indicate that the intact dynein contains ~26% α-helix. The separated β heavy-chain complex and its ATPase-containing amino-terminal domain (fragment A) both have spectra resembling that of intact dynein, and they appear to contain 32% and 23% α-helix, respectively. The carboxy-terminal domain of the β heavy chain (fragment B) and the separated α heavy chain have significantly different spectra; however, they each appear to contain 26–36% α-helix. These data suggest that dynein does not contain an extensive α-helical domain, such as is found in the carboxy-terminal rod region of the other motor proteins myosin and kinesin.

Dynein ATPases play a central role in the motility of cilia and sperm flagella, as well as in some forms of cytoplasmic microtubule-based motility (Gibbons, 1981, 1988). In the axonemes of cilia and flagella, dynein constitutes the inner and outer arms on the doublet microtubules where its function is to generate localized sliding between the doublets by coupling the chemical cycle of ATP binding and hydrolysis with cyclic detachment and reattachment to successive sites along the adjacent doublets. Cytoplasmic dyneins are believed to play a role in vesicle transport in nerve axons (Schnapp & Reese, 1989; Schroer et al., 1989) and may also be involved in mitosis (Pratt, 1984).

Electron microscopy has demonstrated that soluble outer arm dynein has a structure consisting of two or three globular heads, each ~15 nm in diameter, that are connected to a common base by a flexible stem 25–30 nm long (Johnson & Wall, 1983; Goodenough & Heuser, 1984). The examination of the dynein arms in situ on the axonemes has shown that their fixed structural attachment to the A subfiber of each doublet microtubule involves the basal region of their flexible stem and that the heads form the ATP-sensitive site of interaction with the B subfiber of the adjacent doublet microtubule. The cytoplasmic dynein from brain has a similar two-headed electron microscopic structure (Vallee et al., 1988).

Analysis of polypeptide composition has indicated that axonemal dyneins are complex proteins with 9–10 subunits of 3 distinct categories of size. The outer arm dynein from sea urchin sperm flagella is comprised of one α and one β heavy chain of ~470 kDa each, a set of three intermediate-size chains of 76–122 kDa, and at least four light chains of 15–25 kDa (Gibbons et al., 1976; Mocz et al., 1988). The outer arm dyneins of *Chlamydomonas* flagella and *Tetrahymena* cilia contain three heavy-chain subunits (Johnson, 1985; Piperno & Luck, 1979). In all cases, the number of distinct heavy chains is equal to the number of globular heads visible by electron microscopy, which strongly suggests that each globular head/stem unit corresponds to a single heavy-chain polypeptide, with each of these units having a similar tadpole-shaped structure. Direct evidence to support this hypothesis has been obtained by electron microscopy of the separated α and β heavy-chain subunits of outer arm dynein from sea urchin sperm flagella (Sale et al., 1985). The separated β heavy-chain complex is sufficient to produce microtubule motility in an in vitro motility assay system (Sale & Fox, 1988; Vale et al., 1989).

Following the initial observations of Ogawa and Mohri (1975), recent work from this laboratory has shown that

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prolonged tryptic digestion of soluble dynein in a low-salt medium produces two well-defined fragments, termed fragments A and B, which both derive principally from the β heavy chain and are separable by sucrose density gradient centrifugation (Ow et al., 1987). The amino-terminal domain of the β chain, fragment A, retains the ATPase activity of the intact β chain, but has lost its ability to rebind to dynein-depleted flagellar tubules; it is composed principally of 195- and 130-kDa peptides (Ogawa & Mohri, 1975; Tang et al., 1982; Mocz et al., 1988). The carboxy-terminal domain, fragment B, has no ATPase activity and consists of a single 110-kDa peptide. Taken together, fragments A and B account for more than 90% of the mass of the β heavy chain.

In order to understand how dynein acts as an ATP-driven motor to produce microtubule sliding and vesicle transport, it will be important to know the detailed polypeptide conformation of the various structural domains of the dynein arm. Since no amino acid sequence information is yet available, circular dichroic (CD) spectroscopy represents a unique opportunity to examine the secondary structure of the dynein arms and their subfractions. In this paper, we report such analyses of intact outer arm dynein, of the separated α and β/IC heavy-chain complexes, and of tryptic fragments A and B of the β heavy chain. Our data indicate that dynein has no extensive domain of high α-helix content, unlike the other cytoskeletal motor proteins myosin and kinesin. This finding will be useful in interpreting future more detailed conformational models of the complete dynein ATPase molecule when amino acid sequence data become available.

Materials and Methods

Dynein Preparation. Outer arm dynein was extracted from sperm flagella of the sea urchin *Tripneustes gratilla* as described previously (Bell et al., 1982). The extracted dynein was precipitated with 60% saturated ammonium sulfate and then dialyzed for 24 h with three changes against either a low-salt medium containing 7 mM 2-mercaptoethanol, 0.5 mM EDTA, and 10 mM HEPES/NaOH, pH 7.0, or an acetate medium with the same composition plus 0.45 M sodium acetate and 2.5 mM $MgSO_4$. Dynein in acetate medium was further purified by zonal centrifugation on 5–20% sucrose gradients (Tang et al., 1982) and dialyzed into 5 mM Tris/H_2SO_4, pH 7.5, and 1 mM $MgSO_4$ before use.

Preparation of Separated Subunit Fractions. The α heavy chain and β/IC complex were prepared from low-salt-dialyzed dynein samples by sucrose density gradient centrifugation as described previously (Tang et al., 1982). The pooled fractions containing the separated α and β heavy chains were dialyzed into 5 mM Tris/H_2SO_4 buffer, pH 7.5.

Preparation and Purification of Fragments A and B. Fragments A and B were obtained by digesting the low-salt-dialyzed dynein (2 mg/mL) with TPCK-trypsin (Cooper Biochemicals) at 20 °C, with an enzyme to substrate ratio of 1:15 by weight (Ow et al., 1987). After 60 min, the digestion was stopped by adding a 10-fold weight excess of soybean trypsin inhibitor. The digest was then loaded onto 5–20% sucrose gradients prepared in the low-salt medium and centrifuged for 16 h at 34 000 rpm in a Beckman SW 41 rotor. The gradients were separated into 17 fractions (fraction 1 at the top), from which fragments A and B were pooled as fractions 9–11 and 4–6, respectively.

For further purification, the fractions containing fragment A were loaded onto a DEAE-Sephacel column (1 × 18 cm) that had been equilibrated with the standard low-salt buffer. The column was washed with 1.5 column volumes of the same buffer, and then the proteins were eluted with 5 column volumes of a linear gradient of 0.2–0.6 M NaCl at a flow rate of 15 mL/h. One-milliliter fractions were collected and assayed for ATPase activity and protein concentration by the Bradford method. Fractions were subsequently analyzed on 6.5% polyacrylamide gels of Dreyfuss composition (Dreyfuss et al., 1984). Fragment A was eluted from approximately 0.36–0.44 M NaCl. Fragment B was purified in essentially the same way, except that the elution was performed with a linear gradient of 0.3–0.9 M sodium acetate, and it eluted from approximately 0.6 to 0.72 M salt. In both cases, the pooled samples were dialyzed into 5 mM Tris/H_2SO_4 buffer, pH 7.5, and used within 2 days.

Amino Acid Analysis. Proteins were precipitated with 10% trichloroacetic acid prior to hydrolysis. The precipitate was first washed with water and then with 80% acetone. Subsequently, the precipitated material was suspended in 100% acetone, transferred to hydrolysis tubes, and evaporated to dryness. Samples were hydrolyzed for 24 and 72 h in 6 M HCl, 1% mercaptoethanol, and 0.05% phenol, in the absence of air. The amino acid analyses was performed on a Beckman 120 analyzer. Serine and threonine were determined by extrapolation to zero hydrolysis time. Cysteine and methionine were measured as cysteic acid and methionine sulfone, respectively, after oxidation with performic acid. Valine and isoleucine were determined on 72-h hydrolysates. The molar ratio of tryptophan to tyrosine was determined spectrophotometrically in 6 M guanidine hydrochloride (Edelman, 1967), with the absolute amount of tryptophan then being calculated from the known tyrosine content.

Circular Dichroic Spectroscopy. CD spectra were recorded at 1-nm intervals over the wavelength range 190–300 nm, using a Cary Model 61 spectropolarimeter at room temperature. Samples (0.02–0.1 mg/mL) were placed in a 1-mm pathlength cell. All spectra were measured in triplicate with four independent preparations. The net spectrum of each protein fraction was obtained by subtracting buffer base lines as appropriate. The CD data were expressed as the mean residue ellipticity, θ, in units of degrees centimeter squared per decimole, using mean residue weights of 113.5 and 113.0 for fragments A and B, respectively, obtained from their amino acid compositions. For the whole dynein, α chain, and β/IC complex, a value of 113.3 was used. Protein concentrations were determined spectrophotometrically by an absolute method based upon the difference in absorbance at 235 and 280 nm (Whitaker & Granum, 1980). In order to confirm the validity of this method, independent measurements of protein concentration for dynein were also made by quantitative amino acid analysis. The two procedures agreed within 7%.

Secondary Structural Analysis. The measured data points covering the range 190–240 nm were analyzed after smoothing with a five-point cubic least-squares filter (Savitzky & Golay, 1964). Estimates of the apparent secondary structural parameters α-helix, β-sheet, β-turn, and undefined structure were computed by using a least-squares curve-fitting procedure to reference spectra derived from published data for standard proteins. This fitting was first performed with the constraints that the fraction of each structure should be positive and that the sum of the fractions for all the secondary structures should be unity (Chang et al., 1978). The results obtained were then compared with those from an unconstrained least-squares fit with no constraint on the sum of fractions or on negative values (Yang et al., 1986). Spectra measured over the wavelength range 190–240 nm contain nearly 4% of freedom (Hennessey & Johnson, 1981; Manavalan & Johnson, 1987), which in principle permits estimation of the approximate fraction of all

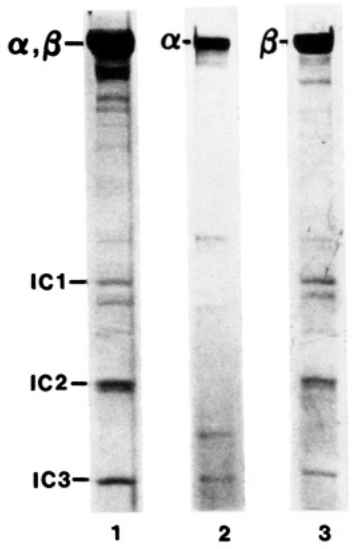

FIGURE 1: Gel electrophoresis patterns showing separation of α and β/IC heavy-chain subunits of outer arm dynein. Sucrose gradient purified outer arm dynein (15 μg) was loaded on lane 1. The α and β heavy chains were separated as described under Materials and Methods. Purified α heavy chain (15 μg) was loaded on lane 2. Purified β/IC fraction (15 μg) was loaded on lane 3. The α and β heavy chains (α,β) and intermediate chains 1, 2, and 3 (IC1, IC2, IC3) are indicated on the left side of the figure.

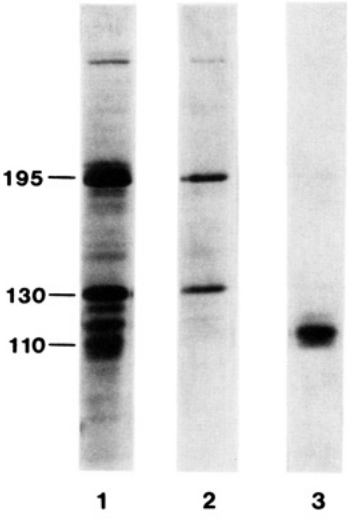

FIGURE 2: Gel electrophoretic patterns showing purification of fragments A and B. Twelve milligrams of dynein (2 mg/mL) was dialyzed against 5 mM HEPES/NaOH, pH 7.0, for 36 h and then digested in the same medium with trypsin at a ratio of 1:15 w/w for 60 min. The digested material (lane 1) was loaded onto 5–20% sucrose gradients prepared in the low-salt medium and centrifuged at 34000 rpm in a Beckman SW41 rotor for 16 h. Pooling of appropriate gradient fractions, followed by further purification on DEAE-Sephacel columns, yielded pure fragment A (lane 2) and fragment B (lane 3). Molecular weights ($\times 10^{-3}$) are shown on the left. Note that fragment A consists principally of 195- and 130-kDa peptides; 15 μg of digest and 7.5 μg of purified proteins were loaded on each lane.

four structural classes. However, in practice, although the predictions of α-helix are generally accurate regardless of the analytical procedure, the estimates for the other secondary structural parameters are sensitive to the procedure, and they may or may not be statistically significant in an uncharacterized protein (Yang et al., 1986; Manavalen & Johnson, 1987). Accordingly, we present values only for the α-helical content in this paper.

The reference CD spectra of 15 globular proteins of known secondary structure were taken from Yang et al. (1986), with a value of 10.4 for the average number of residues per helical segment. As indicated under Results, a subset of these reference proteins was used to improve the degree of fit in some cases. The normalized standard deviation (NRMSD) was used as a goodness-of-fit parameter (Brahms & Brahms, 1980). NRMSD values of less than 0.1 may indicate a successful prediction; values in the range of 0.1–0.2 predict that the calculated structure is generally consistent with the actual structure; values greater than 0.2 indicate disagreement between experimental and calculated data (Mao et al., 1982; Johnson, 1988). Calculations were performed on an AST Premium/286 computer using a program written in Turbo Pascal.

Results

We have analyzed the circular dichroic spectra of intact outer arm dynein, and of its separated α heavy-chain and β/IC complexes, as well as those of tryptic fragments A and B of the β/IC complex. Although the outer arm dynein and its separated heavy-chain fractions were purified by sucrose density gradient centrifugation, it should be noted that whole dynein and the β/IC complex are multisubunit proteins and contain an inherent 20–25% contribution from the intermediate and light-chain subunits present in addition to the α and β heavy chains (Figure 1). Fragments A and B of the β/IC complex were further purified by ion-exchange chromatography in order to free them from small peptides released during tryptic digestion and from the trypsin/inhibitor complex. The resultant preparations of fragments A and B appeared virtually homogeneous by polyacrylamide gel electrophoresis (Figure 2).

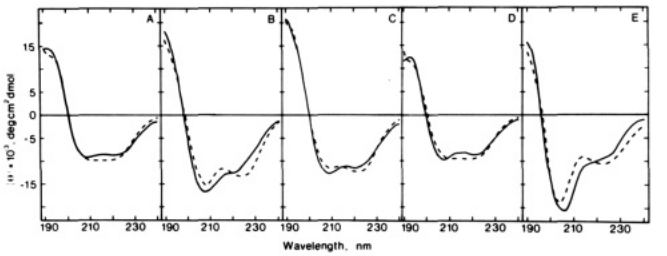

FIGURE 3: Far-ultraviolet circular dichroic spectra of dynein and its derivatives. (A) Intact dynein; (B) α heavy chain; (C) β/IC complex; (D) fragment A of β heavy chain; (E) fragment B of β heavy chain. Proteins were prepared in 5 mM Tris/H_2SO_4 buffer, pH 7.5; for intact dynein, 1 mM $MgSO_4$ was also present. Spectra were obtained with a 1-mm path-length cell at room temperature. Mean residue ellipticities, [θ], are expressed on the basis of a mean residue weight of 113.5 for fragment A, 113 for fragment B, and 113.3 for the other proteins, as calculated from their amino acid compositions. Solid lines show the averaged spectra of four independent preparations for each sample. Dashed lines show calculated spectra obtained by assuming the proportions of secondary structure obtained by constrained least-squares fitting, with the full set of standard proteins for intact dynein, β/IC heavy chain, and Fragment A, and with the subsets of standard proteins for α heavy chain and fragment B.

The CD spectra obtained are shown in Figure 3. All the spectra display a high positive value at 190 nm, a crossover in the vicinity of 199 nm, and two minima at approximately 208 and 222 nm, with a plateau or broad shoulder in between. However, there are substantial differences in the magnitudes of the individual CD maxima and minima, and especially in the ratios at 208 and 222 nm. In spite of their individuality, the observed spectra all generally resemble those of such typical globular proteins as lactate dehydrogenase, subtilisin BPN′, thermolysin, papain, Staphylococcal nuclease, ribonuclease A, lysozyme, and bovine pancreas trypsin inhibitor (Yang et al., 1986). Qualitative comparison of the observed spectra to those for typical proteins of the α/β type that have intermixed

Table I: α-Helix by Circular Dichroism[a]

	constrained	unconstrained	sum	NRMSD
dynein	0.25	0.27	0.76	0.091
α heavy chain	0.28	0.36	1.28	0.150
β/IC complex	0.32	0.32	1.05	0.075
fragment A	0.22	0.23	0.87	0.073
fragment B	0.26	0.29	1.11	0.175

[a] The ellipticity data in Figure 3 were interpreted by the method of Chang et al. (1978), either with or without the constraint that the sum of secondary structure fractions is to unity. Values shown are expressed as fractions and are derived from the mean spectra of four separate preparations of each sample. The standard deviation for the α-helical content of four preparations of each fraction was 0.01 in all cases. Values for the α heavy chain were calculated by utilizing a subset of reference spectra consisting of papain, subtilisin, Staphylococcal nuclease, ribonuclease A, and lysozyme for fragment A heavy chain, and the same subset was utilized for fragment B except that subtilisin was replaced by bovine pancreatic trypsin inhibitor. The column headed "sum" gives the total secondary structural components obtained by unconstrained fitting for each protein sample. NRMSD indicates the normalized root mean square deviation (Brahms & Brahms, 1980).

segments of α-helix and β-sheet, and those for $\alpha+\beta$ proteins that have separate α-helix- and β-sheet-rich regions (Manavalan & Johnson, 1983), supports the validity of this assignment.

The fractional compositions of secondary structure present in dynein and its subfractions were estimated by both constrained and unconstrained least-squares fitting of each of the observed spectra to a calculated composite spectrum derived from a standard reference set of CD spectra of 15 proteins whose proportions of secondary structure are known from X-ray crystallography (Yang et al., 1986).

The calculated and experimental curves for intact dynein, the β/IC complex, and fragment A appear visually to have a good fit (Figure 3), and this is confirmed by their NRMSD values of 0.073–0.091 (Table I). The estimated percentages of α-helix derived from the constrained and unconstrained fits were in very close agreement, with about 26% α-helix in intact dynein, 32% in the β/IC complex, and 23% in fragment A. In the unconstrained fits, no value for any of the secondary structure classes was negative or greater than 1, and the sums of secondary structures are as close to unity as typically found in analyses over this range of wavelengths (Manavalan & Johnson, 1987).

The fits of the experimental spectra for the α heavy chain and for fragment B with spectra calculated from the complete set of 15 standard proteins were less satisfactory, and had NRMSD values exceeding 0.2 (data not shown). In order to improve the degree of fit for these dynein fractions, we generated new reference spectra from subsets of 5 of the 15 standard proteins that had spectra with the lowest NRMSD from the observed spectra. The fit of the resultant calculated composite spectra to the observed spectra of the α heavy chain and fragment B was substantially improved (Figure 3) and predicts percentages of α-helix of 28–36% for the α heavy chain and 22–23% for fragment B (Table I). The corresponding values of NRMSD of 0.15–0.17 are improved from those for spectra derived by using the complete set of 15 standard proteins, although still less good than those for the other dynein fractions. Although this use of a subset of standard proteins improves the degree of fit between the calculated and observed spectra, the estimated percentages of α-helix do not differ substantially from those obtained with the complete set of 15 standard proteins (data not shown), in accord with previous reports of the insensitivity of the predicted α-helical content to the procedure used for curve fitting (Yang et al., 1986; Manavalan & Johnson, 1987).

The major finding is that intact dynein and the subfractions examined all contain only a moderate percentage of α-helix.

Discussion

In considering the above values for the contributions of the various dynein subfractions to the secondary structure of the whole outer arm dynein, the first question to be addressed is whether the conformation of the whole structure is equivalent to the weighted average of its parts. Any substantial discrepancies would suggest that the procedures used to separate the subfractions cause changes in secondary structure. The values of 26% α-helix in the intact dynein, 28–32% in the separated α and β heavy chains, and 22–36% in tryptic fragments A and B of the β heavy chain are probably nearly equivalent within the accuracy of the circular dichroism technique. This suggests that each of the dynein fractions examined contains approximately the same percentage of α-helix as is present in whole dynein and that this aspect of the conformation is little affected by the separation into subfractions. The values of 22–36% α-helix are typical of the majority of globular α/β and $\alpha+\beta$-type proteins (Chang et al., 1986).

The CD spectrum of fragment B and to a lesser extent that of the α heavy chain, however, exhibit an atypical ratio of the intensities of the CD bands at 208 and 222 nm, and the best fits to the experimental spectra that can be obtained with selected subsets of the standard proteins have a moderately high NRMSD of ~0.16. The predictions for α-helix content are probably accurate, since the constrained and unconstrained computations both yield similar values. The inability to obtain a tight fit to the observed spectra of the α heavy chain or of fragment B by using subsets of the reference proteins suggests that these portions of the dynein may contain a distinctive structural motif that is not represented in the reference protein set (Manavalan & Johnson, 1987). Such a motif may occur in the flexible tail portions of the dynein heavy chains, for unlike the corresponding tail portions of myosin and kinesin, they are each constituted principally from a single heavy chain (Sale et al., 1987). However, more detailed discussion must await the availability of amino acid sequence data.

The hypothesis that fragments A and B may correspond to the head and tail portions, respectively, of the tadpole-shaped β/IC subunit was originally formulated on the basis of their sedimentation and enzymatic behavior (Ow et al., 1987). Fragment A has a frictional ratio (f/f_0) of 1.3 and sediments as a more compact, less asymmetric particle than the complete tadpole-shaped β-chain complex which has a frictional ratio of 1.9 (Ow et al., 1987). The aggregation state of fragment B and therefore its native molecular weight are not yet known, but its sedimentation coefficient of 5.7 S is smaller than that of any other dynein fragment reported, which makes any extensive aggregation unlikely. The current paper supports the above hypothesis, for the estimates of secondary structure suggest that fragment A is a globular polypeptide containing about 22% α-helix and that fragment B may contain an unusual structural motif such as might correspond to the structural organization of the tail domain.

The secondary structures of the separated α and β heavy chains appear somewhat different from one another, as are their ATPase and hydrodynamic properties (Tang et al., 1982). At least part of the difference in their CD spectra may be due to the contribution of intermediate chain 1 to the pure β heavy-chain spectrum. This view is supported by the observation that a reconstructed heavy-chain spectrum calculated from the weighted-average spectra of fragments A and B

resembles that of the α heavy chain more closely than that of the β/IC complex (data not presented).

At the electron microscopic level of resolution, the structure of axonemal dynein from sperm flagella has some resemblance to that of the other mechanochemical energy-transducing proteins, myosin and kinesin (Johnson, 1985; McIntosh & Porter, 1989). Each of these motor proteins appears to be composed of a pair of globular heads joined to an elongated tail, with the site of ATP binding and hydrolysis being located in the globular head. Notwithstanding this superficial similarity, a variety of evidence strongly suggests that the structural organization of the heavy polypeptide chains in dynein differs substantially from that of those in myosin and kinesin. For example, the nucleotide binding site in dynein involves a 70–100-kDa region near the middle of each of the ~470-kDa heavy chains (Mocz et al., 1988; King & Witman, 1987), whereas the corresponding site in myosin and kinesin involves a 50–80-kDa region located near or at the amino terminus (McLachlan, 1984; Korn et al., 1987; Kuznetsov et al., 1989; Yang et al., 1989). The present CD study emphasizes the structural difference of dynein from kinesin and myosin. Our data have revealed no extended α-helical domain in the dynein heavy chains and suggest that the carboxy-terminal domain (fragment B) of the dynein heavy chain contains more nonhelical structure than α-helix, in marked contrast to myosin and kinesin where the elongated carboxy-terminal tail domains that join the globular heads are believed to consist of a two-stranded coiled-coil α-helix with one strand derived from each heavy chain (McLachlan, 1984; Yang et al., 1989). This conclusion is supported by electron microscopy showing that the elongated tail extending from each globular head in a dynein molecule appears to be formed mostly from one single heavy chain (Sale et al., 1985), as well as by the fact that it has not so far been possible to isolate a highly helical fragment from tryptic digests of ethanol-treated dynein by the procedure used routinely to isolate the helical tail of myosin (G. Mocz, unpublished results). Thus, while dynein, kinesin, and myosin show striking similarities in their general morphology of multiple globular mechanochemical heads attached to an elongated tail, it seems likely that the tail domain in dynein has a distinctive secondary structure.

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