Localization of an Intermediate Chain of Outer Arm Dynein by Immunelectron Microscopy*

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We have used immuno-electron microscopy to determine the location of an intermediate chain in the isolated outer arm dynein from Chlamydomonas flagella. When the purified αβ dimer of the outer arm was incubated with antibodies recognizing two distinct epitopes on its 69-kDa intermediate chain and then negatively stained and examined by electron microscopy, both antibodies appeared to have bound to the base of the Y-shaped stem that connects the two heads of the particle. These results indicate that this intermediate chain-light chain complex, it is likely that this entire assemblage is located at the base of the particle. Thus, these polypeptides are in a potentially important position with regard to the ATP-insensitive (structural end) binding of dynein to microtubules and to dynein-dynein interactions within the axoneme.

Dyneins are mechanochemical transducers which utilize the hydrolysis of ATP to translocate objects along microtubules (Warner et al., 1989). The inner and outer dynein arms generate the force that produces interdoublet microtubule sliding within cilia and flagella and are directly responsible for the motility of these organelles (Warner et al., 1989). In addition, dynein isoforms have been identified in the cytoplasm of a wide variety of cells and tissues, where they appear to be involved in the translocation of vesicles and particles, and perhaps chromosomes, along microtubules (Warner and McIntosh, 1989). Hence, knowledge of the fundamental molecular structure of dynein will be very important for understanding a wide range of cellular processes.

These enzymes are highly complex (Witman, 1989); for example, the outer arm from Chlamydomonas flagella, one of the most thoroughly characterized dyneins, contains 15 distinct polypeptides with a combined mass of ~1.7 MDa (King and Witman, 1989). This dynein is composed of α, β, and γ subunits; the α and β subunits are isolated together as an αβ dimer, whereas the γ subunit dissociates from the other two during purification and is obtained separately (Piperno and Luck, 1979; Pfister et al., 1982). Each subunit consists of a single heavy chain (the α, β, and γ chains; 480, 440, and 415 kDa, respectively) associated with one or more light chains (8–22 kDa). The β subunit also contains two intermediate chains of 78 and 69 kDa (King and Witman, 1989; King et al., 1986; Pfister et al., 1982), here termed IC78 and IC69.

When examined by electron microscopy, dyneins are seen to have two or three (depending on source) globular heads connected by stems to a common base (Goodenough and Heuser, 1984; Johnson and Wall, 1983; Witman et al., 1983). To understand the molecular organization of dynein it is essential to know the locations of the various chains relative to the structural domains. Models for the arrangement and locations of some of the polypeptides within dynein have been proposed previously (Bell and Gibbons, 1982; Gibbons, 1988; King and Witman, 1989; Mitchell and Rosenbaum, 1986; Vallee et al., 1989; Witman, 1989); these have been based on several lines of evidence, including mass analysis, subfractionation, proteolytic fragmentation, photoaffinity labeling, and hydrodynamic behavior (Bell and Gibbons, 1982; Johnson and Wall, 1983; King and Witman, 1988; King et al., 1989, 1990; Moes et al., 1989; Ow et al., 1985; Sale et al., 1986; Tang et al., 1982; Witman et al., 1983). However, in no case has the substructural location of any dynein intermediate or light chain been determined directly.

In this report, we utilize monoclonal antibodies 1869A and 1869F, which react specifically with different epitopes on IC69 (King et al., 1985), to locate this polypeptide within the isolated Chlamydomonas outer arm dynein by immuno-electron microscopy. The results indicate that this molecule and several additional intermediate and light chains with which it associates are at the base of the dynein. This is the first immuno-electron microscopic localization of any component within the overall dynein superstructure and leads to a detailed model for the arrangement of the chains within this complex molecular motor.

EXPERIMENTAL PROCEDURES

Purification of Dynein-Antibody Complexes—Outer arm dynein was extracted from Chlamydomonas flagella with 0.6 M NaCl and the αβ dimer purified by sucrose density gradient centrifugation (King et al., 1986; Pfister et al., 1982). Monoclonal antibodies 1869A and 1869F were isolated from hybridoma culture supernatant by affinity chromatography on protein A-Aff-Gel (Bio-Rad). Approximately 0.2 μg of αβ dimer and 70 μg of antibody were incubated together for ~5 h and then applied to a 12.4 ml 5%–20% sucrose gradient made in 30 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, and 150 mM NaCl and centrifuged for ~13,5 h at 160,000 × g (ω2 = 6.51 × 105 rad2/s). Fractions of 0.7 ml were collected from the bottom of the gradient.

Gel Electrophoresis and Protein Blotting—Fifty-μl aliquots of sucrose gradient fractions and other samples were analyzed by electrophoresis in 0–24 m glycerol, 5–15% acrylamide gels containing sodium dodecyl sulfate (King et al., 1986). Gels were stained either with Coomasie Brilliant Blue or by the silver stain procedure of Merrif et al. (1981). Alternatively, separated proteins were electrophoretically blotted to

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1The abbreviations used are: IC, intermediate chain; LC, light chain.
nitrocellulose and stained with Amido Black or to reveal monoclonal antibody reactivity as described previously (King et al., 1986; Otter et al., 1987).

**Electron Microscopy—Sucrose gradient-purified dynein-antibody complexes** were diluted with Tris-buffered saline (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) to a final protein concentration of 20–30 μg/ml. A thin carbon film was floated from the supporting mica sheet onto the surface of the protein solution. After 5–10 s, the film was withdrawn, floated completely free of the mica onto a pool of 1% uranyl acetate (aqueous), and picked up using acetone-washed, uncoated 400-mesh copper grids (Marchese-Ragona et al., 1988). For some experiments, the carbon film was pretreated with 1% tryptophan (aqueous) and rinsed with water (Roux et al., 1987) prior to the application of dynein. All samples were examined in a Philips CM-10 electron microscope operating at 80 kV and calibrated by means of a replica grating.

**RESULTS AND DISCUSSION**

Outer arm dynein was extracted from Chlamydomonas flagella with 0.6 M NaCl and the αβ dimer subsequently purified by sucrose density gradient centrifugation. Monoclonal antibodies 1869A and 1869F were obtained from hybridoma culture supernatant (Fig. 1a). Immunoblot analysis indicates that both antibodies react with IC 69 and do not recognize any other dynein component (Fig. 1b). Purified antibodies were then added either individually or together to the αβ dimer preparation and incubated for several hours. The amount of antibody added was in large molar excess over the amount of dynein in order to reduce the number of dynein duplexes formed.

Antibody-dynein complexes were separated from unbound antibody by a second sucrose density gradient centrifugation step (Fig. 2). Antibody not associated with dynein sedimented in these gradients at ~6 S, whereas αβ dimer complexed with either 1869A (Fig. 2) or 1869F (not shown) sedimented at ~21 S. Antibodies appeared to be present in approximately stoichiometric amounts with the αβ dimer. The fact that both of these antibodies form stable complexes with native dynein indicates that the epitopes recognized are located at the surface of the dynein particle.

When antibodies 1869A and 1869F were added to the same dynein preparation, both appeared to associate in approximately stoichiometric amounts with the αβ dimer (not shown). The complexes formed were heterodisperse and sedimented at ~12–21 S. This reflects the formation of a variety of multimeric dynein-antibody aggregates and indicates that the 1869A and 1869F epitopes are spatially separate such that binding of one antibody does not sterically hinder binding of the other.

The sucrose density gradient-purified antibody-dynein complexes then were examined by negative stain electron microscopy (Figs. 3 and 4). In the absence of antibody, the αβ dimer appeared as two globular heads (~12 nm in diameter) joined by thin stems to a common base (Figs. 3a and 4, top row), as described previously (Johnson and Wall, 1983; Witman et al., 1983). The stems and base were pleiomorphic and often were not readily distinguishable in negatively stained images. In samples treated with both antibodies (Fig. 3b), or separately with antibody 1869A (Fig. 4, middle row) or 1869F (Fig. 4, bottom row), a different morphology was observed. In these cases, the dynein heads were joined via stems to a greatly enlarged base. The additional basal material was not observed in any sample not reacted with these particular antibodies; for example, addition of antibody 1878A (which recognizes IC 78 only in the denatured form (King et al., 1986)) did not enhance the size of this domain. Thus, the increase in size of the basal region is due to antibody binding at the 1869A and 1869F epitopes. In samples incubated with antibody 1869A or 1869F, we occasionally observed two αβ dimers joined at their bases (not shown); presumably, these structures represented two dynein particles cross-linked by a
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FIG. 3. Survey electron micrographs of dynein particles (a) without bound antibody, and (b) complexed with monoclonal antibodies 1869A and 1869F. Scale bar, 100 nm; arrows on b indicate some of the dynein molecules with antibodies bound to the base.

single bifunctional antibody. Association of dynein particles as a result of cross-linking was more evident in samples incubated with both antibodies (see Fig. 3b), where the potential for formation of multimeric aggregates existed. These results indicate that IC 69 is located at the base of the dynein particle.

Immunoprecipitation of detergent-dissociated dynein has shown that IC 69 is tightly associated with IC 78 and light chains of 20 and 11 kDa (Mitchell and Rosenbaum, 1986). In some experiments, we occasionally observed that the anti-IC 69 antibodies caused dissociation of the intermediate chains from the αβ dimer; in this case the two intermediate chains and light chains of 8, 11, and 14 kDa cosedimented as a discrete particle (not shown). Thus, in *Chlamydomonas* outer arm dynein, as in the outer arm dynein from sea urchin (Tang et al., 1982; Sale et al., 1985; Witman et al., 1991) and trout sperm flagella (King et al., 1990), two intermediate chains and several light chains associate together to form a discrete intermediate chain-light chain (IC-LC) complex. The localization of IC 69 to the base of the dynein particle strongly suggests that the other components of the IC-LC complex similarly are located within this structural domain (Fig. 5). We expect this to be a general architectural feature of all dyneins which contain intermediate chains.

Analysis of the αβ dimer by scanning transmission electron microscopy has demonstrated that the mass of each globular head domain is ~375 kDa (Witman et al., 1983); thus, each head is too small to accommodate an entire heavy chain and must contribute mass to both the head and stem regions of the particle. In addition, each heavy chain is tightly associated with a single light chain (Mitchell and Rosenbaum, 1986; Pfister and Witman, 1984; Pfister et al., 1982); these probably are located within the head domains, as occurs in myosin (Warrick and Spudich, 1987). In this case, the α and β heavy chains together would provide ~250 kDa to form the stems and, in association with the IC-LC complex, the basal domain of the dynein particle.

Electron microscopic analysis of intact axonemes has shown that the outer dynein arms overlap such that the head domains of one arm associate with the basal region of the next (Goodenough and Heuser, 1984). This, together with the observations reported here, indicates that the IC-LC complex of one dynein arm is close to and might interact directly with the heavy chains of the adjacent arm (Fig. 6). Interaction between the head and basal domains of different dynein molecules could provide a mechanism for the spatial and temporal regulation of dynein arm function necessary for ciliary and flagellar motility (Warner, 1989; Witman, 1990). In this regard, it is interesting that the α heavy chain is phosphorylated *in vivo* at multiple sites (King and Witman,
Fig. 4. Montage of electron microscopic images of single dynein particles negatively stained with uranyl acetate. Scale bar, 50 nm. Top row, dynein without added antibody; middle row, dynein complexed with antibody 1869A; bottom row, dynein complexed with antibody 1869F. An interpretive diagram at the end of each row depicts the structures observed.

Fig. 5. Model for the structure of the αβ dimer from Chlamydomonas flagellar outer arm dynein. The diagram indicates the locations of various components within the particle and the predicted mass of each structural domain. Analysis by scanning transmission electron microscopy has demonstrated that each head domain has a mass of ~375 kDa and that the entire particle has a mass of ~1.22 MDa (Witman et al., 1983). Therefore, the stem and base of the complex must account for ~470 kDa. The data presented here demonstrate that IC 69 is located at the base of the particle and strongly support the localization of IC 78 and six light chains (20, 14, 14, 11, 8, and 8 kDa (Pfister et al., 1982)) to this same domain. Together, these molecules account for ~220 kDa, so the heavy chains must contribute no more than ~250 kDa to the non-head regions of the complex. The latter value represents an upper limit due to uncertainty in the stoichiometry of the 8-kDa light chains (King and Witman, 1989; Pfister et al., 1982).

1989; Piperno and Luck, 1981) and that IC 78 apparently contains a nucleotide binding site (King et al., 1989; Pfister et al., 1985). Interactions between the intermediate chains of one arm and the heavy chains of the next arm also could be responsible for the apparent cooperative binding of dynein to microtubules (Haimo and Fenton, 1984).

Finally, localization of the IC-LC complex to the base of the flagellar dynein particle raises the possibility that one or more of these components are involved in binding of the dynein arm to the A-microtubule of the axonemal outer doublets. As flagellar and cytoplasmic dyneins appear to differ mainly in their IC-LC components (Paschal et al., 1987), it also is quite possible that these latter polypeptides function as adaptors to attach the dynein motor to different intracellular structures.

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