ATP-Insensitive Interaction of the Amino-Terminal Region of the β Heavy Chain of Dynein with Microtubules†

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ABSTRACT: The ATP-insensitive microtubule-binding site of dynein has been investigated by limited proteolysis of sea urchin sperm flagellar axonemes. Mild tryptic digestion cleaved the dynein β chain at either of two principal cleavage sites, generating two sets of complementary peptides. Inclusion of ATP in the digestion medium had no effect on the generation of these primary fragments. Sucrose density gradient separation and immunostaining with monoclonal antibodies against epitopes on the β chain showed that extraction of the digested axonemes with 1–3 mM ATP solubilizes the peptides located at the carboxy-terminal end of the original heavy chain. The solubilization of the peptides containing the amino end required the presence of 0.6 M NaCl and was not affected by ATP. While the outer arm dynein is in situ on the axoneme, the N-terminal 125-kDa domain of the β chain was not digested by trypsin, whereas in soluble dynein this domain becomes rapidly degraded. These data suggest that the N-terminal domain of the β chain is involved in its ATP-insensitive attachment to microtubules and support the hypothesis that the N-terminal 125-kDa peptide corresponds to the flexible tail of the dynein molecule seen in electron micrographs.

The dynein ATPases are heterooligomeric proteins that, depending on species, contain either two or three (α, β, γ) heavy polypeptide chains with molecular masses larger than 400 kDa and several chains of intermediate (70–125 kDa) and low (15–25 kDa) molecular mass (Gibbons, 1988; Vallee & Shpetner, 1990). Among the heavy chains, the separated β chain is of special interest because it is able to interact with microtubules and translocate them in in vitro motility assays (Sale & Fox, 1988; Vale et al., 1989), and because its complete amino acid sequence has recently been determined (Gibbons et al., 1991; Ogawa, 1991). The amino acid sequence contains the typical consensus motif for four P-loops that are usually considered indicative of nucleotide-binding sites and has no homology to the sequence of other families of motor proteins. The hydrolytic ATP-binding site has been identified in the midregion of the peptide chain, in agreement with earlier data on the structural and functional organization of the β chain (Mocz et al., 1988; King & Witman, 1988; Inaba et al., 1991a,b). The sequence determination of the β chain has shown that its polarity is the reverse of that deduced earlier by assaying acetyl residues present in the chain (Mocz et al., 1988; Gibbons et al., 1991), possibly because the β chain contains acetylated amino acids located elsewhere than the N-terminus. The relative positions of the photolytic and proteolytic peptides in this reversed polarity, as well as the relative location of epitopes recognized by specific monoclonal antibodies, are all consistent with the sequence data and can serve as a point of departure for further studies.

In all cell types, the solubilized dynein has a characteristic bouquetlike appearance with two or three globular heads attached by elongated tails to a common base (Johnson & Wall, 1983; Witman et al., 1983; Sale et al., 1985). In situ, axonemal dynein appears as a more compact structure, referred to as the dynein arm, which consists of the apposed heads joined to the microtubule by an elongated base that is composed of the compacted tails and at least some of the intermediate chains (Goodenough & Heuser, 1984, 1985; King & Witman, 1990; King et al., 1991).

The dynein heavy chains contain two types of microtubule-binding site. The heads possess the ATP-sensitive sites that interact cyclically with the adjacent microtubule in the process of mechanochemical energy transduction. The other, ATP-insensitive, site is at the base, and it makes a structural attachment to each microtubule (Goodenough & Heuser, 1985). However, there is as yet no evidence regarding which regions of the heavy-chain polypeptides are involved in each of these two microtubule-binding sites. Earlier work from this laboratory, based on sequential extraction of digested axonemes in buffers of different composition, has indicated that the affinity of outer arm dynein to the microtubules is greatly diminished by brief tryptic digestion of the α chain (Bell & Gibbons, 1982). Furthermore, study of the functional substructure of the solubilized β chain by limited proteolysis has shown that the digested β chain is separable into two fragments, A and B, that sediment at 12 S and 6 S, respectively, suggesting that these fragments may correspond to the head and tail domains of the molecule (Ogawa & Mohri, 1975; Ow et al., 1987). The circular dichroic spectra of the two fragments are consistent with this hypothesis, the spectrum of fragment A resembling that of a globular protein and that of fragment B suggesting that it possesses an unusual structural motif such as might correspond to the organization of the elongated tail domain (Mocz & Gibbons, 1990). The head–tail domain structure is not readily apparent from the predicted secondary structure of the β chain, which consists of an α/β structure along its whole length, and only two short regions strongly suggestive of a coiled-coil α-helix (Gibbons et al., 1991).

In vitro translocation studies of dynein and its separated subunits adsorbed onto glass have suggested that structural and ATP-sensitive binding of dynein to the microtubules are both mediated by the α chain (Moss et al., 1992a,b). The isolated β/intermediate-chain (IC)[1] complex fails to bind to

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[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IC, intermediate chain.

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the microtubules in either an ATP-sensitive or an ATP-insensitive manner, whereas the α chain binds in the absence or the presence of ATP. Interactions between the α and β subunits have been shown to change in the presence of ATP (Shimizu & Johnson, 1983; Inaba et al., 1990a,b). Perhaps the conformational changes caused by ATP hydrolysis may be partially responsible for the differences in microtubule-binding affinities between the two subunits. On the other hand, an intermediate chain of dynein has also been shown to be involved in structural binding to the microtubules (King & Witman 1990; King et al., 1991).

In the present study, we extend our previous work (Bell & Gibbons, 1982) on sequential extraction of digested axonemes to investigate the protein–protein interactions in the structural binding site of the dynein β chain to the microtubules. Results obtained by employing limited tryptic proteolysis of axonemes in conjunction with sucrose gradient centrifugation and immunoblotting indicate that the amino-terminal domain, fragment B, engages in an ATP-insensitive attachment to the microtubules, either directly by contact or indirectly through its association with other subunits of the dynein.

MATERIALS AND METHODS

Isolation of Axonemes. Flagellar axonemes were obtained from sperm of the sea urchin *Tripneustes gratilla* in a standard isolation buffer containing 0.1 M NaCl, 4 mM $MgSO_4$, 0.1 mM EDTA, 7 mM 2-mercaptoethanol, and 5 mM HEPES/NaOH, pH 7.0, as described previously (Bell et al., 1982). The axonemes were washed twice with the same buffer before digestion experiments. To obtain reproducible results, it was found important to remove sperm heads completely from the preparation.

Digestion with Trypsin. Limited proteolysis was performed by adding trypsin (TRTPCK, Worthington) at a trypsin: protein ratio of 1:2500 (w/w) to the standard medium containing 10–15 mg/mL axonemal protein. In experiments in which ATP was added to the digestion medium, the axonemes were incubated with ATP for 5 min prior to addition of trypsin, and the free Mg^{2+} concentration was maintained by a buffer containing 0.25 mM EDTA and the appropriate amounts of $MgSO_4$ and ATP. The digestion was allowed to proceed for 60 min at 23 °C and then was stopped by adding a 10-fold weight excess of soybean trypsin inhibitor (Worthington).

Sequential Extraction of Digested Flagellar Axonemes. Axonemes were centrifuged at 12000*g* for 10 min immediately after digestion (referred to as digestion supernatant/pellet in the text). The pellet was washed with the standard isolation medium (wash supernatant/pellet). In two successive extraction steps, the digested axonemes were first extracted with 2 mM ATP in standard medium and centrifuged to yield fractions called the ATP supernatant and pellet, and finally extracted with 0.6 M NaCl in standard medium and centrifuged to yield the fractions called the 0.6 M NaCl supernatant and pellet. The extraction time in each case was 15 min at 4 °C followed by centrifugation at 12000*g* for 10 min. The pellets were resuspended in the same volumes as they had been sedimented from. The supernatants were analyzed by sucrose gradient centrifugation. Samples of all supernatants and resuspended pellets were subjected to SDS–polyacrylamide gel electrophoresis and blotted onto Immobilon.

Zonal Centrifugation. The α and β heavy-chain fractions and related digested peptides were separated by sucrose density gradient centrifugation of each of the supernatants after dialysis into 0.5 mM EDTA and 5 mM HEPES/NaOH buffer, pH 7.0 (Bell et al., 1982).

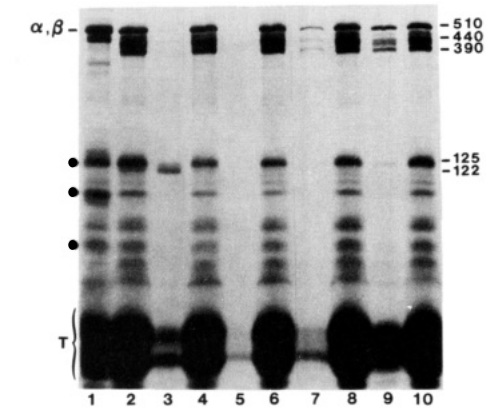

FIGURE 1: Polyacrylamide gel electrophoresis showing sequential washing extraction of digested axonemes. Axonemes (15 mg of protein/mL) were digested by trypsin (1:2500 by mass) at room temperature in 0.1 M NaCl medium for 60 min and subjected to sequential washing and extraction as described under Materials and Methods. Lanes 1 and 2, undigested and digested axonemes. Lanes 3 and 4, digestion supernatant and pellet. Lanes 5 and 6, wash supernatant and pellet. Lanes 7 and 8, ATP supernatant and pellet. Lanes 9 and 10, 0.6 M NaCl supernatant and pellet. To show the partition of the protein components in the different steps of the experiment, the same volume (25 μL) was loaded in each lane. The *numbers* represent approximate molecular masses (in kilodaltons) of the principal cleavage peptides. *Dots* indicate the intermediate chains; T corresponds to the tubulin doublet. Note that in the digested samples three peptides comigrate at ~125 kDa: IC1 + two fragments deriving from the amino and the carboxy end of the β chain, respectively (see Figure 5 and Discussion).

Determination of ATPase Activity and Protein Concentration. ATPase activities of dynein extracts and of gradient fractions were measured at room temperature under the conditions described earlier (Tang et al., 1982). The inorganic phosphate liberated was determined by the method of Fiske and SubbaRow. Protein concentrations were determined by the Coomassie Blue dye-binding assay, using bovine serum albumin as standard.

Gel Electrophoresis and Protein Blotting. Gel electrophoresis in the presence of sodium dodecyl sulfate was performed on 6.5% polyacrylamide gels by the method of Dreyfuss et al. (1984), followed by staining with Coomassie Blue R-250. Quantitation of band intensities was performed on gels that were dried onto filter paper by excising the bands of interest and extracting the dye with 75% dimethyl sulfoxide (Gibbons et al., 1987). Digestion rates for dynein heavy and intermediate chains as well as for tubulin were obtained by fitting the decay of dye intensity in the bands to a single-exponential function. For antibody staining, protein bands in the gels were electroblotted onto a poly(vinylidene difluoride) membrane (Immobilon, Millipore Corp.) and stained with monoclonal antibodies directed toward known regions of the β heavy chain of dynein (Mocz et al., 1988; Gibbons et al., 1991).

RESULTS

Tryptic Digestion of Axonemes. Preliminary experiments have shown that tryptic digestion of axonemes at room temperature in physiological salt medium (0.1 M NaCl) cleaves all the major protein components at different rates. At trypsin:axonemal protein ratios of 1:100 to 1:10 000, the fastest digested protein components are the dynein heavy chains, followed at approximately half the rate by cleavage of the intermediate chains. The tubulin becomes digested

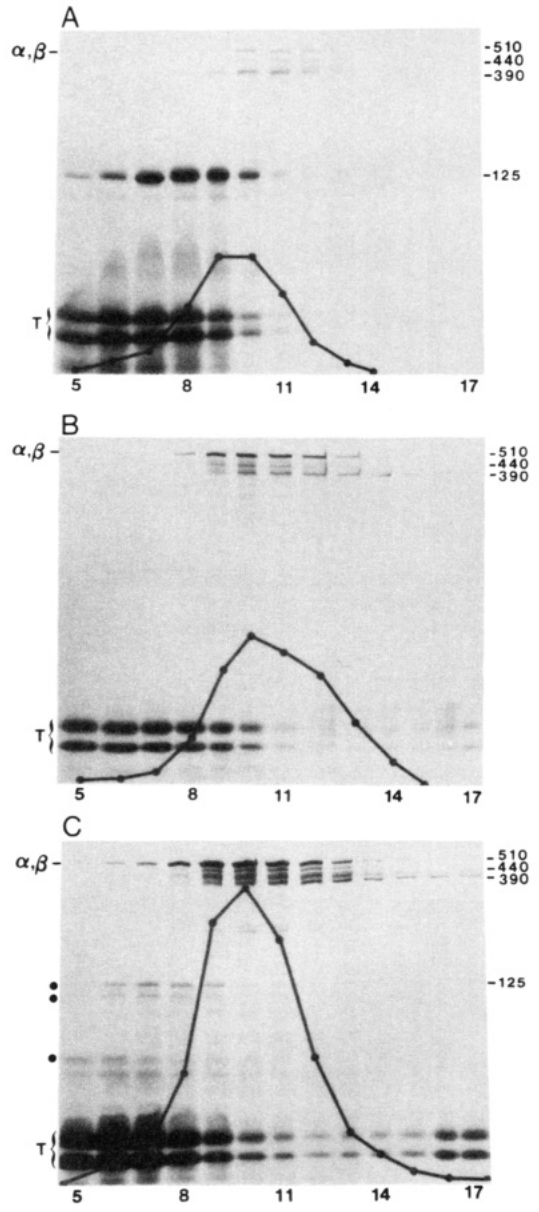

FIGURE 2: Separation of peptides of digested axonemes by sucrose density gradient centrifugation. Digestion (panel A), ATP (panel B), and 0.6 M NaCl (panel C) supernatants from the experiment shown in Figure 1 were dialyzed against a low-salt medium and centrifuged on 5–20% sucrose gradients to separate the α- and β-chain peptides of dynein (Tang et al., 1982). (Fraction 1 is the top of the gradient. The β chain is found principally in fractions 8–10 and the α chain in fractions 15–17.) Portions of each gradient fraction were used for assay of the Triton-activated ATPase activity. Plots of the ATPase activity are superimposed on the photograph of the gel. Pooled ATPase activities of 0.083, 0.098, and 0.196 μmol of $P_i \cdot min^{-1} \cdot mL^{-1}$ were recovered from gradients A, B, and C, respectively. These activities correspond respectively to 50, 59, and 118% recovery relative to a 0.6 M extract of undigested dynein under the same conditions. The high level of ATPase recovery of the three supernatants is largely related to augmentation in ATPase activity upon tryptic digestion of the β chain (Bell & Gibbons, 1982; Ow et al., 1987).

about 1 order of magnitude more slowly. In order to prevent overdigestion of the axonemes, which generally occurs at trypsin:protein ratios above about 1:500 and results in solubilization and extensive further digestion of the dynein heavy chains, a ratio of 1:2500 (by weight) was chosen for all subsequent experiments. When axonemes are digested for 60 min at this ratio of trypsin to protein, only about 5–10% of the tubulin is lost from the pellet in the course of digestion. Approximately 40% of the total heavy-chain polypeptides remain intact, while the rest are converted to slightly smaller

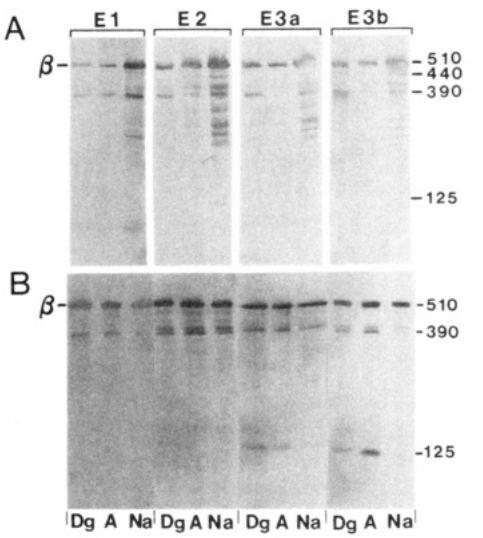

FIGURE 3: Distribution of epitopes in the dynein β chain after digestion of axonemes in 0.1 M NaCl medium. Fractions 8–10 (12S peak) from gradients A, B, and C in Figure 2 were combined as separated digestion (D_g), ATP (A), and 0.6 M NaCl (Na) extracts and were then subjected to epitope mapping (panel A) together with the pellet fractions from Figure 1, lanes 4, 8, and 10 (panel B). Strips of each immunoblot were reacted with monoclonal antibodies against epitopes E1, E2, E3a, and E3b on the β heavy chain (for location and identification of the epitopes, see Figure 5). The higher sensitivity of the antibody staining highlights some peptides that are not visible in the samples stained with Coomassie Blue (Figures 1 and 2).

molecular mass peptides in the range 390–460 kDa, with simultaneous appearance of distinct smaller peptides of ~122–125 kDa (Figure 1). Upon centrifugation of the digest, nearly all the 122-kDa peptides together with a small fraction of the high molecular mass peptides remain in this digestion supernatant (Figure 1, lane 3). Sucrose density gradient centrifugation of this supernatant suggests that the 390–460-kDa peptides are derived from the β chain, all sedimenting at ~12 S (Figure 2A) and reacting with antibodies against epitopes on the β chain (Figure 3A). The prominent 122-kDa peptide is not recognized by any of these antibodies. Its early formation, that was observed in control experiments, suggests that it may derive principally from the α heavy chain. Digestion of axonemal and solubilized dynein with trypsin and chymotrypsin indicates that the proteolysis of the α chain proceeds with a generally faster rate than that of the other heavy-chain components of the axoneme (Bell & Gibbons, 1982).

Comparison of the peptide patterns in parallel digests performed in the absence and presence of 1–3 mM ATP indicated that ATP has no effect on the early stages of digestion, nor on the solubility properties of the individual peptides in the subsequent extraction steps.

Extraction by ATP. Supernatants resulting from centrifugation of the washed and digested axonemes in standard medium contain very small amounts of peptides, showing the stability of the preparation (Figure 1, lanes 5 and 6). However, upon subsequent addition of ATP (1–3 mM) to the standard medium, approximately 30% of the remaining intact heavy chains and their related high molecular mass digestion products appear in the supernatant after centrifugation (Figure 1, lanes 7 and 8). The dependence of peptide release upon ATP concentration over the range 0.01–3 mM showed a hyperbolic profile with half-maximal ATP concentration occurring at 0.3 mM and a plateau leveling off at >1 mM (data not shown). Sucrose density gradient analysis of this ATP supernatant indicates that a 390-kDa fragment of the α chain or inner arm

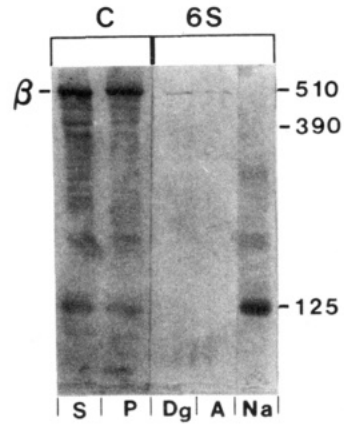

FIGURE 4: Polypeptide composition of the 6S fractions of digested axoneme by epitope mapping. Fractions 5–7 (6S peak) from gradients A, B, and C in Figure 2 were combined as separated digestion, ATP, and 0.6 M NaCl extracts and were then reacted with monoclonal antibodies against epitope 3a. The first two lanes show unfractionated 0.6 M NaCl supernatant and pellet, respectively, that serve as electrophoretic controls. Conditions and abbreviations are as in Figure 3.

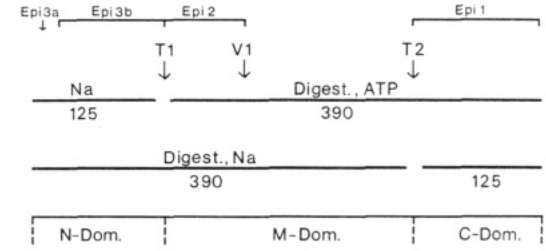

FIGURE 5: Linear map of the principal cleavage sites of dynein β heavy chain in the axoneme. The diagram shows the relative position of the primary tryptic fragments. T1 and T2 represent the principal sites of cleavage. The V1 site of vanadate-mediated photocleavage is also indicated as a point of reference (Gibbons et al., 1987). The numbers are approximate molecular masses in kilodaltons. Peptides found in the digestion, ATP, and 0.6 M NaCl supernatants are indicated by Digest, ATP, and Na, respectively. Dom stands for domains of dynein: N, amino terminal; M, middle; C, carboxy terminal (Ogawa, 1991). The map is shown with the polarity determined from the sequence of the β chain (Gibbons et al., 1991). The epitopes of E1, E2, E3a, and E3b (clones 6-31-24, C-241-2, 4-69-14, and C-26-5, respectively) have been localized previously (Piperno, 1984; Mocz et al., 1988).

dynein, and some of the β-chain peptides in the 390–460-kDa range, became solubilized by the ATP (Figure 2B). Little or no intermediate chain was released. Indirect evidence that the 390-kDa peptide, running far down in the gradient, is not of β-chain origin was found in control experiments in which no reaction was observed with monoclonal antibodies specific for epitopes on the β chain. Addition of ATP to undigested axonemes has no significant solubilization effect on dynein, with less than 3% of the total axonemal ATPase activity being solubilized in the presence of 3 mM ATP.

Immunoblotting of the sucrose gradient-separated ATP extract shows that the β-chain-related peptides contain either or both epitopes 1 and 2 (for a map of epitopes on the heavy chain, see Figure 5). Of particular importance is the lack of reaction of any of the cleavage peptides of the supernatant fraction with the antibodies specific for epitopes 3a and 3b. Together with a fraction of apparently intact heavy chain, all the epitope 3a- and 3b-bearing peptides remain in the pellet fraction with masses of ~390 and 125 kDa. This indicates that the N-terminal region of the β heavy chain remains strongly associated with the microtubules, regardless of the presence of ATP.

Extraction by 0.6 M NaCl. When the digested axonemes are extracted with 0.6 M NaCl, subsequent to the extraction with ATP, all species of the remaining dynein peptides, including the 390-kDa α-chain or inner arm peptide and the intermediate chains, are represented in the supernatant (Figure 1, lanes 9 and 10; Figure 2C). The ATPase in the extract represents ~50% of the total combined ATPase in the three supernatants investigated in this study. Especially notable is the solubilization of the epitope 3a- and 3b-bearing peptides which had not been solubilized by ATP in the previous step, with little of these peptides remaining in the final pellet (Figure 3B). The presence of 3a and 3b epitope-bearing peptides of 390 kDa strongly suggests that trypsin can cut at either the T1 or the T2 site of the β chain to generate either an N-terminal or a C-terminal 390-kDa peptide, respectively.

Figure 3A illustrates the 12S fractions of the sucrose gradients to show the solubility properties of the large molecular mass peptides. In these samples, the 125-kDa peptide appears only with very low intensity originating from minor contamination with the 6S fraction. Figure 4 shows the corresponding 6S fractions of digestion, ATP, and NaCl supernatants. Almost all of the 125-kDa peptide is found in the NaCl supernatant.

DISCUSSION

The early cleavage pattern described here for the β heavy chains of dynein bound to the axoneme generally resembles that reported previously for digestion of solubilized dynein under low-salt conditions (Ow et al., 1987), with formation of a 390-kDa peptide (containing epitopes 1 and 2) and a 125-kDa peptide (containing epitopes 3a and 3b) by cleavage at or near the T1 site. However, while bound to the axoneme, the β chain also becomes cleaved at a comparable rate near the T2 site, generating a second set of similar size fragments with different epitope distribution, i.e., a 390-kDa peptide containing epitopes 2 and 3a/3b and a complementary 125-kDa peptide that is not recognized by the antibody specific for epitope 1 and thus is not easily traceable (Figure 5). This latter peptide may correspond to the 124-kDa high-salt peptide of solubilized dynein (Mocz et al., 1991) and very likely comigrates with the corresponding α-chain peptide of nearly similar size. Our data indicate that, under the mild proteolytic conditions used, the cleavage of a given β heavy chain occurs either at the T1 site or at the T2 site, but not at both. This suggests either that the primary clevage at one of these sites alters the tryptic accessibility of the other or that the β chain exists in two states on the axoneme. β heavy-chain peptides cleaved at both the T1 and T2 sites are observed in a significant amount only at approximately 5 times higher concentrations of trypsin or after much longer digestion times. Antibody staining has shown that cleavage also occurs within the two terminal regions of the β chain in the axonemes, generating fragments with molecular masses higher than 390 kDa (Figure 3). Complete details of the formation of these latter peptides are not yet clear, but are of marginal relevance to the main points of this paper.

The initial cleavage of the axoneme-bound α chain and/or inner arm dynein occurs at a single cleavage site, generating 390- and 122-kDa peptides, apparently almost identical in mass to the β-chain fragments mentioned above. This cleavage is 80–90% complete under the enzymatic conditions used, whereas the β chain is digested more slowly with ~40% of it remaining intact. The solubility properties of these two peptides differ from the similarly sized peptides of the β chain, with the 122-kDa fragment being completely released into

the digestion supernatant and the 390-kDa fragment appearing both in the ATP and in the 0.6 M NaCl extract. This indicates that the 390-kDa fragment of the other dynein heavy chains may correspond to the similar size β fragment that is generated by cleavage at the T2 site and that it may have a role in the structural microtubule binding. However, in the absence of antibodies reacting with specific regions of these chains, it is not possible to identify their binding region at this time.

The present data show that the N-terminal region (fragment B) of the β chain interacts with axonemal microtubules in an ATP-insensitive manner, for fragment B and related larger fragments are not released by ATP from the digested axoneme, but require a high concentration of salt to solubilize them. On the other hand, fragments deriving from the carboxy end (fragment A) of the β heavy chain are easily solubilized by ATP, indicating that the salt-sensitive association site between these latter peptides and the microtubules has been partially removed or altered by digestion. It is important to point out that not only the large peptides containing the region corresponding to fragment B but also fragment B itself is largely preserved in the digest of the axonemes, in contrast to the fragment B of soluble dynein which is not stable to tryptic digestion at physiological salt concentration (Mocz et al., 1991). Its presence in the digested axonemes as an intact peptide provides strong support for it being protected from proteolysis by interaction with the microtubules. Since both epitopes 3a and 3b are preserved in this peptide, its site of interaction with microtubules probably lies toward the middle and N-terminal regions of fragment B, where these epitopes are located (Mocz et al., 1991; Gibbons et al., 1991). Within the limitations of the proteolytic approach, our data lend strong support to the suggestion made previously (Ow et al., 1987) that fragment B corresponds to the tail domain of the molecule. Furthermore, the data that the peptides corresponding to fragment A can be solubilized from the digested axonemes by ATP show that the head domain, composed of the middle and C-terminal regions of the β chain, contains the ATP-sensitive binding site for microtubules.

It cannot be decided at this time whether the structural attachment occurs directly to the microtubules or indirectly through the α heavy chain or intermediate chain. However, the fact that much of the β chain released from the digested axonemes by ATP appears undigested favors an indirect association of the β chain with the microtubules. This hypothesis is supported by the localization of the intermediate chains at the base of the dynein heterooligomer heavy chains (King & Witman, 1990; King et al., 1991) and by the recent observations that the isolated β/IC complex does not form a structural attachment with cytoplasmic microtubules (Moss et al., 1992a,b).

The present proteolytic study did not reveal ATP-insensitive structural association on any other portion of the β chain of dynein with the microtubules, although it cannot be excluded that such interactions exist since the outer arm dynein possesses relatively extensive contacts with the microtubules. Further analysis employing a different chemical approach is needed for a more complete characterization of the protein–protein interactions at the structural and ATP-sensitive microtubule-binding sites.

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