A Map of Photolytic and Tryptic Cleavage Sites on the β Heavy Chain of Dynein ATPase from Sea Urchin Sperm Flagella

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Abstract. NH_2-terminal analysis of the α and β heavy chain polypeptides (M_r > 400,000) from the outer arm dynein of sea urchin sperm flagella, compared with that of the 230,000- and 200,000-M_r peptides formed upon photocleavage of dynein by irradiation at 365 nm in the presence of vanadate and ATP, shows that the NH_2 termini of the intact chains are acetylated and that the 230,000- and 200,000 M_r peptides constitute the amino- and carboxy-terminal portions of the heavy chains, respectively. Tryptic digestion of the β heavy chain is known to separate it into two particles, termed fragments A and B, that sediment at 12S and 6S (Ow, R. A., W.-J. Y. Tang, G. Mocz, and I. R. Gibbons, 1987. *J. Biol. Chem.* 262:3409–3414). Immunoblots against monoclonal antibodies specific for epitopes on the β heavy chain, used in conjunction with photoaffinity labeling, show that the ATPase-containing fragment A is derived from the amino-terminal region of the β chain, with the two photolytic sites thought to be associated with the purine-binding and the γ-phosphate-binding areas of the ATP-binding site spanning an ~100,000 M_r region near the middle of the intact β chain. Fragment B is derived from the complementary carboxy-terminal region of the β chain.

DYNEIN ATPase is the energy-transducing enzyme responsible for generating the sliding movement between tubules that underlies the beating of cilia and flagella of eukaryotes, and is also responsible for some forms of microtubule-based motility within the cytoplasm (7, 17, 22, 28). In spite of much work demonstrating the importance of dynein in cell function, rather little is known about its detailed chemical structure, partly because of its very high molecular mass and partly because of the relatively small quantities available for study. When solubilized from cilia or eukaryotic flagella, outer arm dynein has a native molecular mass (depending on species) of 1.3–2.0 × 10^6, with a polypeptide composition consisting of either two or three distinct heavy chain subunits with M_r > 400,000,[1] termed α and β, (and γ when present), as well as several intermediate chains of 85,000–122,000 M_r and at least four light chains of 15,000–25,000 M_r (1, 8, 13, 27). Separation of the heavy chain subunits has shown that they each possess a nucleotide-binding site and are capable of hydrolyzing ATP (24, 25, 35, 37). Examination of the structure of solubilized dynein by electron microscopy has shown that it consists of a small number of globular units, ~15 nm in diameter, joined together by slender flexible stems ~15 nm long (11, 14, 29, 41). In all cases, the numbers of globular heads has been equal to the number of heavy chain polypeptides present in that preparation, suggesting that each of the heavy chains in solubilized dynein has a basically tadpole-shaped tertiary structure consisting of a globular head with a short flexible tail. This hypothesis is supported by observations on the separated α and β heavy chains from outer arm dynein of sea urchin sperm flagella (29).

Attempts to obtain proteolytic fragments of dynein that possess subsets of its properties were begun by Ogawa and Mohri (19), who demonstrated that tryptic digestion of dynein from sea urchin sperm flagella in a low salt medium leads to formation of a semi-stable particle, termed fragment A, that sediments at 12S and retains the ATPase activity of intact dynein but that has lost its ability to rebind to dynein-depleted flagellar tubules. Fragment A was reported to have a native M_r of 370,000 and to be composed principally of 195,000- and 130,000-M_r peptides. More recently, our laboratory has shown that fragment A is derived solely from the β heavy chain, and that its formation is accompanied by that of a complementary 6S particle, fragment B, which lacks ATPase activity and consists of a peptide of 110,000 M_r. On the basis of their sedimentation properties, it was suggested that fragments A and B may correspond to the head and tail, respectively, of the tadpole-shaped particle seen by electron microscopy in preparations of the intact β heavy chain (21, 29).

Mapping of the functional domains in dynein heavy chains, as well as eventual determination of their amino acid sequence, will be greatly facilitated by recent work showing that the heavy chains of outer arm dynein from sea urchin sperm flagella can be cleaved at either of two specific sites,

1. Because of the absence of standard proteins of sufficiently high molecular mass, the molecular mass of the dynein heavy chains has been difficult to measure by gel electrophoresis. Recent determinations using summation of the molecular masses of their specific photolytic and proteolytic cleavage peptides yield values of 428,000–475,000 D (see Discussion).

identified as V1 and V2, by irradiation at 365 nm in the presence of the ATPase inhibitor vanadate (V_i).[2] Photolysis of this outer arm dynein at the V1 site, which occurs upon irradiation in the presence of ATP and low micromolar concentrations of V_i, forms HUV1 and LUV1 peptides with M_rs of 230,000 and 200,000, respectively (10, 16). Photolysis at the V2 site occurs upon irradiation in the presence of Mn^{2+} and 100 μM V_i, in the absence of ATP, and results in formation of HUV2 and LUV2 peptides with M_rs of ~255,000 and 175,000 (9, 36). The V1 and V2 sites, which are separated by ~100,000 M_r along the length of the heavy chains, are believed to be located on separate loops of the tertiary structure, both of which constitute part of its ATP-binding site.

The data in this paper establish the orientation of the dynein heavy chains with respect to the V1 photocleavage site by demonstrating the presence of an acetylated NH_2-terminal residue in the intact heavy chains and in the HUV1 peptides. A series of monoclonal antibodies specific for epitopes on the β heavy chain has been used to locate the V1 and V2 sites with respect to each other and to the principal sites of tryptic cleavage that separate putative functional domains of the β chain.

The monoclonal antibodies used were generously donated by Dr. Gianni Piperno of The Rockefeller University (New York). Their characterization and use in identifying dynein in the cytoplasm of sea urchin embryos have been described previously (26). The properties of monoclonal antibodies against dynein from *Chlamydomonas* have been described by King et al. (15).

Materials and Methods

Dynein Preparation

Outer arm dynein was obtained from sperm flagella of the sea urchin *Tripneustes gratilla* (1). When desired, the α and β heavy chains were separated by dialysis against a low salt medium, followed by sucrose density gradient centrifugation (35). Photoaffinity labeling of the heavy chains with [α-^{32}P]8-azidoadenosine 5'-triphosphate (8-N_3ATP) was performed as described by Lee-Eiford et al. (16).

Electrophoresis

Analytical electrophoresis was performed on SDS polyacrylamide gels of Dreyfuss composition as described previously (5, 10, 39).

Preparative electrophoresis was performed on SDS polyacrylamide gels of Dreyfuss composition with a 3-mm thick separating gel containing 7% acrylamide and 0.063% bisacrylamide. 2 mg protein were applied per square centimeter of loading surface and the gels were run at 150 V for 5 h. Protein bands were located in the gel by briefly incubating it with 0.08% Coomassie Brilliant Blue G-250 (3). The bands containing the desired peptides were excised, ground, and extracted three times for 24 h with 4 vol of a solution containing 0.1% SDS, 0.1% 2-mercaptoethanol, 0.5 mM EDTA, and 50 mM Tris-HCl, pH 9.0, at 22 °C. The extracts were filtered and then dialyzed exhaustively against distilled water. After lyophilization, the samples were redissolved in the above buffer and insoluble residue removed by centrifugation. Proteins were then precipitated by 9 vol of acetone. The precipitate was first washed with 80% and then with 100% acetone. The acetone-washed material was used for peptide analysis. Quantitation of peptides was on the basis of dry mass. The yield of preparative electrophoresis was 58 ± 7% (n = 8).

NH_2-terminal Analysis

After electrophoretic separation of intact heavy chains, and of the HUV1 and LUV1 cleavage peptides, 5–10 nmol of each acetone-washed sample were dansylated at pH 8.0 according to Percy and Buchwald (23). The dansylated peptides were hydrolyzed for 4 and 18 h at 105°C in 1 ml of 6 N HCl containing 80 mM 2-mercaptoethanol under nitrogen. 0.5–1.0 nmol of each hydrolyzed sample was analyzed by thin-layer chromatography on polyamide sheets (Schleicher & Schuell, Keene, NH) according to Woods and Wang (42) and Hartley (12). Solvent systems (vol/vol) were (*a*) 1.5% formic acid; (*b*) benzene/acetic acid 9:1; and (*c*) ethyl acetate/methanol/acetic acid, 20:1:1 (reference 4). A second portion of the sample, containing 0.5 nmol of a reference set of dansyl amino acids added to it, was run in parallel in all cases. All chromatography was performed in duplicate.

Acetyl Group Determination

Putative acetylated or formylated NH_2-terminal residues were identified by conversion to 1-acetyl-2-dansyl hydrazine or 1-formyl-2-dansyl hydrazine by the method of Schmer and Kreil (30), as modified by Mocz et al. (18). To minimize contamination with adventitious acetate, the intact dynein heavy chains, as well as the HUV1 and LUV1 cleavage peptides, were prepared in modified solutions containing no added acetate. 10 nmol of each of the electrophoretically purified, acetone-washed peptides were twice redissolved in 0.1 M HCl and lyophilized to remove residual traces of acetate. The same molar quantities of ovalbumin, which has an acetylated NH_2-terminal α-amino group, and of trypsin, which has a free amino-terminal group, were analyzed in parallel as controls. In all cases, the dried residue was heated with anhydrous hydrazine for 18 h at 100°C in a sealed ampoule. Excess hydrazine was removed under reduced pressure. The residue was dansylated in 0.2 M citrate buffer, pH 3.0, containing 50% ethanol, with fivefold excess of dansyl chloride (wt/wt) for 24 h at 37°C. The sample was evaporated to dryness, redissolved in 1 ml water, and extracted three times with an equal volume of chloroform. The combined extracts were concentrated, applied to polyamide sheets, and developed with a solvent of benzene/acetic acid 9:1 vol/vol in the first dimension, and of 1.5% formic acid in the second dimension. The reference substances, 1-formyl-2-dansyl hydrazine and 1-acetyl-2-dansyl hydrazine, were synthesized according to Schmer and Kreil (30). For quantitation, a 25-mm^2 section of each polyamide sheet containing 1-acetyl-2-dansyl hydrazine spot was excised and eluted with 3 ml chloroform for 24 h.

Fluorescence measurements were performed in a spectrofluorimeter (model 8000C; SLC Instruments, Inc., Urbana, IL) with the excitation at 335 nm, and emission recorded over a range of 360–600 nm. The spectra were corrected for solvent blanks. The curves were integrated over a range of 480–600 nm, and their areas compared with those of calibration samples eluted from chromatograms loaded with 0–1.0 nmol of the synthesized standard 1-acetyl-2-dansyl hydrazine. The values obtained for the peptides were corrected for remaining traces of acetate contamination from the reagents by subtracting the value of acetyl derivative in the trypsin blank. On this basis, the recovery of 0.47 mol of 1-acetyl-2-dansyl hydrazine in the ovalbumin standard indicates that the overall recovery from the hydrazinolysis and dansylation was ~50%, which is comparable to that achieved by previous workers using this technique in the 10–100 nmol range (38). Because of the high molecular mass of dynein heavy chains and the limited amount available, we were obliged to use 1-nmol quantities, making substantial blank corrections unavoidable. All preparations were chromatographed and quantitated in duplicate, and replicate values agreed within 5%.

Immunoblotting and Antibody Staining

After electrophoresis, the polyacrylamide gels were soaked in transfer buffer (50 mM Tris, 384 mM glycine, 20% methanol, and 0.01% SDS) for 30 min (15). The gel was then transferred onto either nitrocellulose (type BA83; Schleicher & Schuell, Keene, NH) or PVDF paper (Millipore Corp., Bedford, MA), and the peptide bands were transferred in a transverse electrophoresis apparatus (Bio-Rad Laboratories, Richmond, CA) at 40 V for 15 h and cooled with water.

The nitrocellulose and PVDF blots were processed for antibody staining using reagents from Promega Biotech (Madison, WI). After nonspecific staining had been blocked by soaking in 1% BSA in Tris-buffered saline containing Tween 20 for 30 min, the blots were soaked with an appropriate dilution of the primary monoclonal antibody for 1 h. After washing to remove unbound antibody, the blots were exposed to the secondary antibody (goat anti-mouse IgG conjugated with alkaline phosphatase) for 1 h. Staining was performed with a freshly prepared solution containing 0.33 μg/ml Nitro Blue tetrazolium and 0.16 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate.

The original designations of the hybridoma clones yielding the anti-dynein antibodies used (26) were simplified for ease of description. The

2. *Abbreviations used in this paper*: V_i, inorganic vanadate; 8-N_3ATP, 8-azidoadenosine 5'-triphosphate.

antibodies from clones 6-31-24 and 6-64-14 appeared to recognize the same epitope, which was designated as epitope 1. The epitope recognized by clones C-241-2 and 2-79-2 was similarly designated as epitope 2. The epitope recognized by clone 4-69-14 was designated epitope 3a, and that recognized by clone C-26-2 as epitope 3b.

Trypsin (TPCK treated) was obtained from CooperBiomedical (Malvern, PA), ovalbumin and anhydrous hydrazine were from Sigma Chemical Co. (St. Louis, MO). Other reagents were of standard analytical quality.

Results

Polarity of Dynein Heavy Chains

To determine the polarity of the dynein heavy chains with respect to the sites of V_i-mediated photocleavage, we first attempted to compare the amino-terminal residues of the intact heavy chains and of the HUV1 and LUV1 peptides resulting from photocleavage at the V1 site. Electrophoretically purified dynein heavy chains and their separated HUV1 and LUV1 cleavage peptides were subjected to dansylation at pH 8.0, followed by acid hydrolysis in 6 N HCl (23). Thin-layer chromatography showed the presence of only ε-dansyl-lysine, O-dansyl-tyrosine, dansyl-NH_2, and dansyl-OH in all of the hydrolyzates. No spot characteristic of any α-dansyl amino acid was present in any of the preparations.

Since the amino terminus of most cytoskeletal proteins is blocked by acetylation, we then proceeded to assay equimolar quantities of the intact heavy chains and of the separated HUV1 and LUV1 cleavage peptides for the presence of acetyl or formyl groups by microhydrazinolysis (30). Thin-layer chromatograms showed the green fluorescent spot characteristic of 1-acetyl-2-dansyl hydrazine in the hydrazinolates of the intact heavy chains (Fig. 1) and of the HUV1 peptides, as well as in that of ovalbumin standard. A lesser amount of 1-acetyl-2-dansyl hydrazine was also present in the LUV1 peptides and the trypsin blank. The peak wavelength of the fluorescent emission averaged 515 ± 3 nm for six traces of two samples obtained from intact dynein heavy chains, and 515 ± 2 nm for the corresponding samples from the HUV1 peptides, both in excellent agreement with the average of 517 ± 4 nm obtained with seven traces of the synthesized 1-acetyl-2-dansyl hydrazine. No 1-formyl-2-dansyl hydrazine was detected in the protein samples.

Quantitation of the amount of acetyl derivative in each sample indicates that both the intact dynein heavy chains and their HUV1 cleavage peptides contain close to 1 mol of acetyl per mole of peptide, whereas the LUV1 peptides contain little or no acetyl (Fig. 2). Taken in conjunction with the apparent absence of a free amino terminal in the dynein heavy chains, the data indicate that the amino-terminal residues of the α and β heavy chains are acetylated, and that the HUV1 cleavage peptides correspond to their amino-terminal portions. The apparent absence of a free α-NH_2-group in the LUV1 peptides suggests that the V_i-mediated photocleavage reaction blocks the α-NH_2 group at the new amino terminal created, although we cannot exclude the alternative possibility that this amino terminal is a tryptophan, for this would be destroyed by the acid hydrolysis.

Distribution of Epitopes on Photocleaved Peptides

An electrophoresis gel showing preparations of dynein in which the heavy chains have been photocleaved at either their V1 or V2 site, or at both the V1 and V2 sites is shown in Fig. 3, together with corresponding immunoblots treated with antibodies against epitopes 1, 2, and 3a. Although both the α and β heavy chains are present in the preparations of dynein, their V1 cleavage sites are located at almost identical locations and their V2 sites differ in location by only ~5,000 D on each chain, so that their cleavage peptides appear to coelectrophorese. The antibodies used are specific for the β chain, and show no crossreactivity with the α chain (Tang, W.-J. Y., and I. R. Gibbons, unpublished data). In material cleaved at the V1 site, epitope 1 is located in the HUV1 peptide (230,000 M_r), and therefore lies toward the amino-terminal region of the intact β chain, whereas epitopes 2 and 3a are in the LUV1 peptide (200,000 M_r), and therefore lie

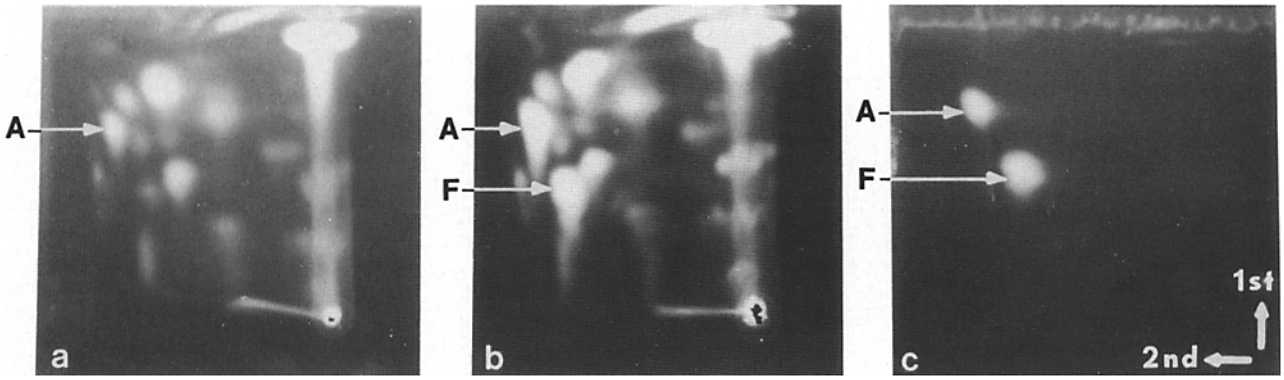

Figure 1. Typical thin-layer chromatograms used for the identification and quantitation of 1-acetyl-2-dansyl hydrazine in sample after hydrazinolysis. The arrows from *A* and *F* indicate spots of 1-acetyl-2-dansyl hydrazine and of 1-formyl-2-dansyl hydrazine, respectively. (*a*) 0.71 nmol of hydrazinolyzed, dansylated, intact α and β heavy chains was loaded onto a thin-layer polyamide sheet near the bottom right corner. The green spot corresponding to 1-acetyl-2-dansyl hydrazine is indicated by the arrow from *A*. The other spots appeared orange or blue, and represent unknown byproducts of hydrazinolysis and dansylation similar to those observed by previous workers using this technique (30). (*b*) 1.0 nmol of the synthesized 1-acetyl-2-dansyl hydrazine and 1.0 nmol of 1-formyl-2-dansyl hydrazine were mixed with the sample before loading onto the thin-layer sheet. Note that the intensity of the spot corresponding to 1-acetyl-2-dansyl hydrazine is enhanced relative to that in *a*. (*c*) Approximately 2 nmol of synthesized 1-acetyl-2-dansyl hydrazine and 1-formyl-2-dansyl hydrazine were loaded onto a thin-layer plate and chromatographed as in *a*. In all cases, two dimensional chromatography was performed as described in Materials and Methods, with the directions of the first and second dimensions being as indicated in the lower right corner of the figure. An ultraviolet lamp with peak emission at 302 nm was used to excite the fluorescence for visual observation and photography.

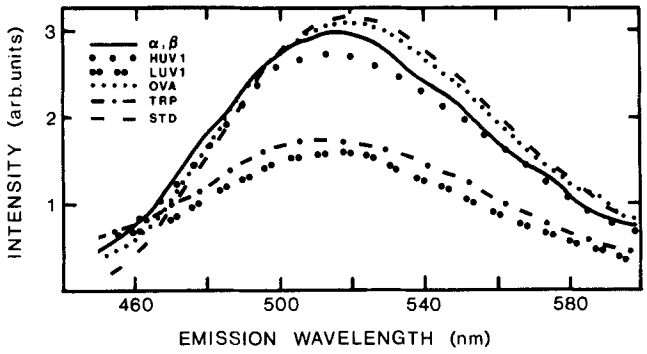

Figure 2. Fluorescent emission spectra of 1-acetyl-2-dansyl hydrazine obtained from various peptides. The traces show typical spectra for samples obtained by eluting the spot of 1-acetyl-2-dansyl hydrazine from thin-layer polyamide chromatograms with chloroform. The hydrazinolyzed, dansylated peptide loaded on each sheet corresponded to 0.71 nmol of each of the following: combined α and β heavy chains (α, β), HUV1 photocleavage peptides (*HUV1*), LUV1 photocleavage peptides (*LUV1*), ovalbumin (*OVA*), and trypsin (*TRP*). Also shown is a trace from 0.8 nmol of authentic 1-acetyl-2-dansyl hydrazine, chromatographed and eluted in the same way (*STD*). The total amount of 1-acetyl-2-dansyl hydrazine recovered from each sample (in mole/mole) was as follows: intact heavy chains, 1.0; HUV1 cleavage peptides, 0.94; LUV1 cleavage peptides, 0.54; ovalbumin, 1.1; and trypsin, 0.63 (averages of two preparations). After correction for reagent blank and for incomplete recovery (see Materials and Methods), these values corresponded to 0.8, 0.7, and −0.2 mol/mol for the intact heavy chains, HUV1 cleavage peptides, and LUV1 cleavage peptides, respectively. The negative value obtained for the LUV1 peptides suggests that the trypsin used as a blank may have contained minor acetate contamination. The slight displacement of the fluorescent peak in the different traces is due to noise in the spectrofluorimeter, and it does not appear in the averaged peak position of replicate scans of the same samples (see Results).

toward the carboxy-terminal region of the intact chain. In material cleaved at the V2 site, epitope 1 lies in the LUV2 peptide (175,000 M_r), which must therefore correspond to the amino-terminal region of the β chain, while epitopes 2 and 3a both lie in the HUV2 peptide (255,000 M_r), corresponding to the carboxy-terminal region of the β chain (Fig. 3).

Previous work has shown that when dynein heavy chains are first cleaved at their V2 site and then subjected to a secondary V1 cleavage, the HUV2 peptide formed by the first cleavage undergoes scission during the second cleavage to form a typical LUV1 peptide and a "daughter peptide" with an apparent M_r of 100,000 (9, 36), while the LUV2 peptide appears unaffected. Immunoblots of such doubly cleaved material show that epitopes 2 and 3a pass from HUV2 into the LUV1 peptide as a result of the second cleavage. As might be expected, epitope 1 remains in the LUV2 peptide.

Taken together, these results indicate that epitope 1 lies within the 175,000-M_r amino-terminal region of the intact β chain, and that epitopes 2 and 3a lie within the 200,000-M_r carboxy-terminal region of the intact chain (Fig. 7). None of the epitopes falls within the daughter peptide of apparent M_r 100,000 that lies in the mid-region of the β chain.

Distribution of Epitopes on Tryptic Peptides

Although in our earlier work we were able to identify the major pathway by which tryptic digestion of the β heavy chain leads to formation of fragments A and B (Fig. 4 and reference 21), the occurrence of two distinct peptides of ~130,000 M_r at different stages of the digestion makes the steps in the pathway difficult to visualize in electrophoretic gels of the preparation as a function of digestion time (Fig. 5 *A*). The steps in this pathway become much more clearly apparent in im-

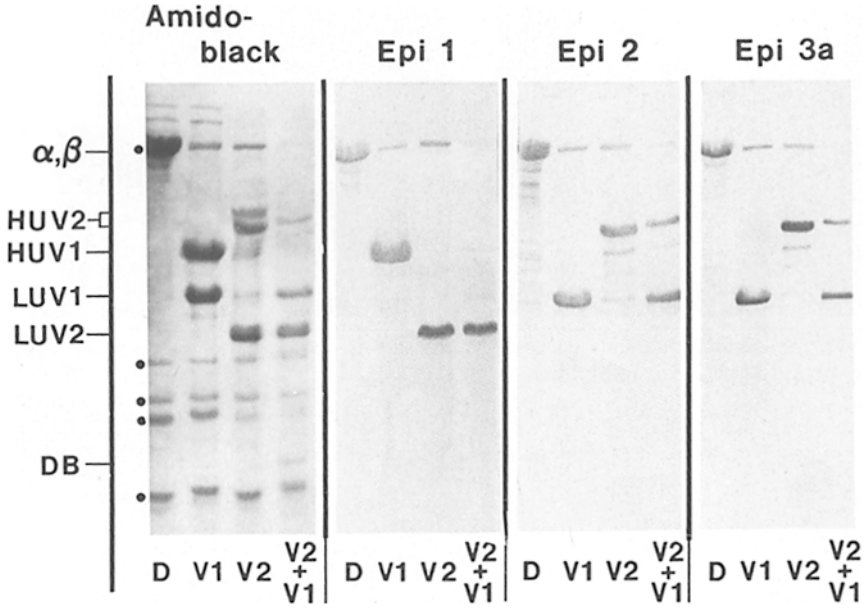

Figure 3. Immunoblots of photocleaved dynein. Four blots onto nitrocellulose paper, each prepared from an identical SDS polyacrylamide gel containing a lane of intact dynein, of dynein photocleaved at the V1 site, of dynein photocleaved at the V2 site, and of dynein photocleaved successively at the V2 and V1 sites. The leftmost blot was stained with amido black to show total protein. The other blots were treated with antibodies against epitopes 1, 2, and 3a, (clones 6-31-24, C-241-2, and 4-69-14, respectively), followed by goat anti-mouse IgG conjugated with alkaline phosphatase, and color development as described in Materials and Methods. The cleavage peptides from the α and β chains coelectrophorese, except for the HUV2 peptides as indicated. The daughter peptide (*DB*) is visualized with amido black staining only, and does not bear any of the epitopes. The dots on the left side indicate (*from top to bottom*): intact α and β heavy chains, a contaminant membrane peptide, dynein intermediate chain 1′, dynein intermediate chain 1, and dynein intermediate chain 2 (I). The protein load was 20 μg per lane in all cases.

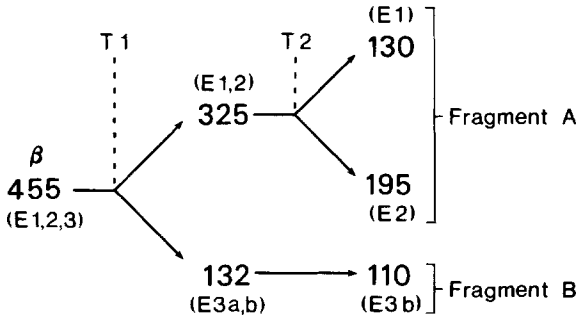

Figure 4. Principal pathway of tryptic digestion of the β heavy chain in a low salt medium. The numbers represent the molecular masses of the tryptic peptides in thousands. T1 and T2 represent the first and second major sites at which trypsin cleaves the β heavy chain under these conditions (21). E1, E2, E3a, and E3b indicate the presence of epitopes 1, 2, 3a, and 3b in the peptides (see Fig. 5 and text).

munoblots of different stages of the digestion, which show the distribution of epitopes among the various tryptic peptides (Fig. 5, *b–d*). These blots show that the initial cleavage at the first major tryptic site, identified as T1 in Fig. 4, cuts the β chain into a 132,000-M_r peptide bearing epitope 3b, and a 325,000-M_r peptide bearing epitopes 1 and 2. Subsequent digestion at the second major tryptic site, identified as T2, cleaves the 325,000-M_r peptide into the 130,000- and 195,000-M_r peptides of fragment A, which bear epitopes 1 and 2, respectively. Nearly simultaneously, a short segment is digested from one end of the first formed peptide of 132,000 M_r, converting it to the 110,000-M_r peptide of fragment B, which continues to bear epitope 3b (Fig. 5 *d*). Epitope 3a is located on the same initial 132,000-M_r peptide as epitope 3b, but is close to one end and becomes lost (data not shown) when this peptide is cleaved to 110,000 M_r.

Combining this information with that given above regarding the distribution of epitopes upon photocleavage of the β chain, we deduce that the T1 site is located 132,000 M_r from the carboxy terminus of the chain, while the T2 site is located 130,000 M_r from its amino terminus (Fig. 7).

Photocleavage sites in Fragment A

Fragment A was prepared from dynein that had been photoaffinity labeled with the ATP analog [α-^{32}P]8-N_3ATP and subjected to photocleavage at its V1 site before digestion with trypsin. Gel electrophoresis comparing fragment A obtained from this photocleaved dynein with that from intact dynein shows that in the cleaved sample the 195,000-M_r peptide of fragment A is cut into peptides of 125,000 and 70,000 M_r (Fig. 6 *A*). The binding site for 8-N_3ATP, which has been located previously on the 195,000-M_r peptide of fragment A (21), is found in the 125,000-M_r peptide after photocleavage (Fig. 6 *B*). There was no indication that photocleavage had other effects on either the speed or pathway of tryptic digestion (data not shown).

To confirm this result, the above experiment was repeated with the V1 photocleavage step being performed after the tryptic digestion to form fragment A. The photocleavage of such preformed preparations of fragment A occurs more slowly than with native dynein, but the result is to cleave the 195,000-M_r peptide into the same peptides of 125,000- and 70,000-M_r (Fig. 6 *C*) that occur when the cleavage is per-

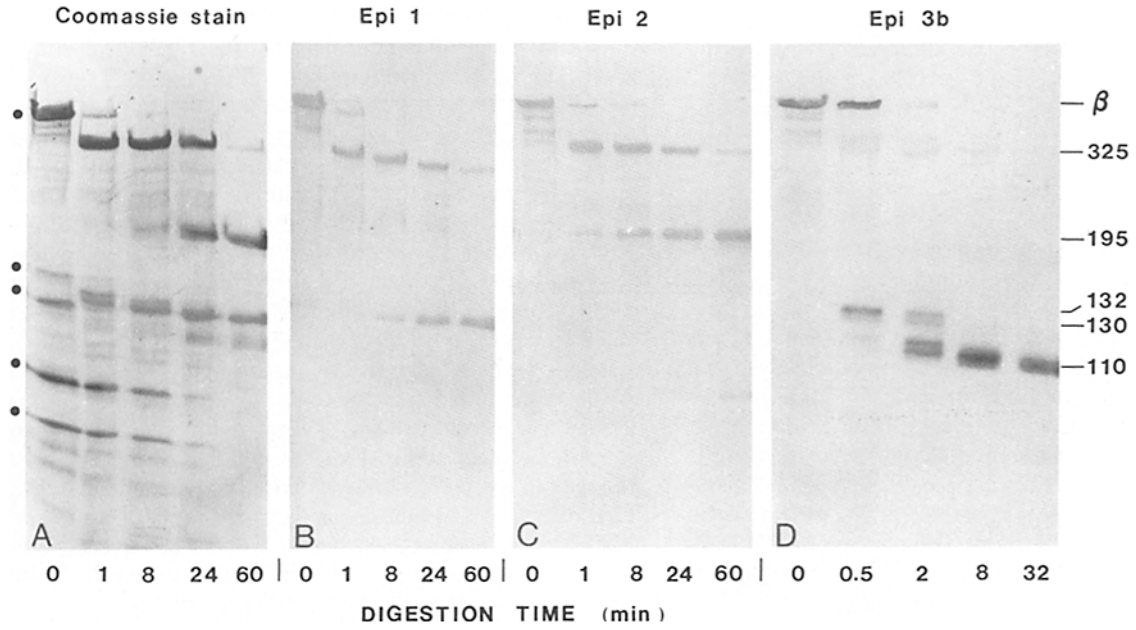

Figure 5. Immunoblots of tryptic digests of isolated β heavy chain as a function of digestion time. Four nitrocellulose blots prepared from SDS polyacrylamide gels, each showing a preparation of the β heavy chain/intermediate chain 1 complex digested with trypsin at a ratio of 1:15 wt/wt for the times indicated. *A*, *B*, and *C* represent the same digest of β chain. *D* comes from a similar preparation digested for somewhat shorter times in order to show the transition of the M_r 132,000 to the M_r 110,000 peptide more clearly. *A* was stained with Coomassie Blue to show total protein; *B*, *C*, and *D* were exposed to antibodies against epitopes 1, 2, and 3b (clones 6-31-24, C-241-2, and C-26-2, respectively) and stained as in Fig. 3. The dots on the left side indicate (*from top to bottom*): β heavy chain, intermediate chain 1′, intermediate chain 1, intermediate chain 2, and intermediate chain 3, which cosediment with the β heavy chain on sucrose gradients (35). 12 μg protein per lane was loaded on the gel for Coomassie Blue staining, and 3 μg per lane on the gels for antibody staining.

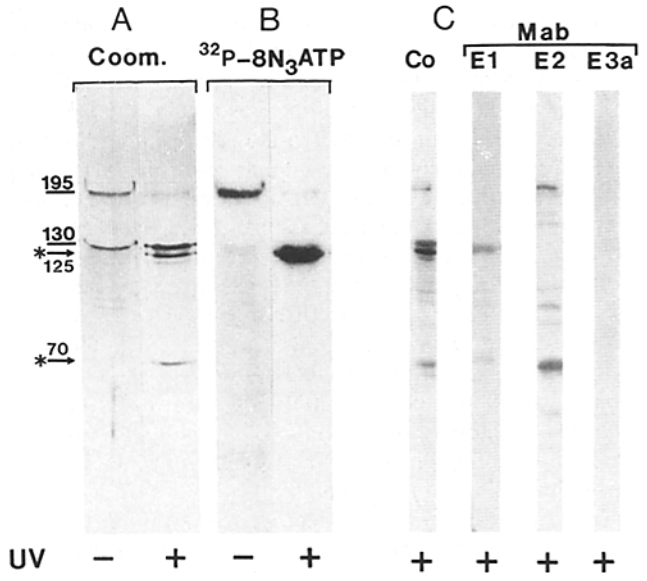

Figure 6. Photoaffinity labeling and immunoblotting of tryptic fragment A subjected to V1 photocleavage. *A* and *B* show a preparation of dynein that was first photoaffinity labeled with [α-^{32}P]8-N$_3$ATP (16) and then photocleaved at the V1 site by irradiation for 30 min in a medium containing 50 μM V_i and 100 μM ATP (10). This preparation and an uncleaved control were then dialyzed into low salt medium, digested with trypsin 1:15 (wt/wt) for 32 min, and subjected to sucrose density gradient centrifugation (21). (*A*) Coomassie-stained electrophoresis gel of fragment A (15 μg) prepared from uncleaved (*left*) and cleaved (*right*) samples of dynein. Note that the 195,000-M_r peptide in the unirradiated sample has been cleaved to a pair of peptides of 125,000 and 70,000 M_r (*asterisks*) in the irradiated sample. (*B*) Autoradiogram of the same gel showing that the 8-N$_3$ATP present in the 195,000-M_r peptide of the uncleaved sample passes into the 125,000-M_r peptide upon photocleavage. *C* shows blots onto PVDF paper of single lanes loaded with preformed fragment A subjected to photocleavage at the V1 site by irradiation for 60 min in the presence of 0.1 mM V_i and 2 mM ATP. (*Co*) Fragment A after staining with Coomassie Blue for total protein (8 μg loaded); (*E1*, *E2*, and *E3a*) replicate lanes (0.8 μg protein loaded) treated with antibody against epitopes 1, 2 and 3a, respectively, as in Fig. 3.

formed before digestion. When the dynein had been photoaffinity labeled with 8-N$_3$ATP before digestion, the label was found in the same 125,000-M_r peptide as above (data not shown). These data indicate that photocleavage of preformed fragment A by irradiation under V1 conditions occurs at the same site as on the intact β heavy chain.

Immunoblotting of the fragment A subjected to cleavage at its V1 site shows that epitope 2 passes from the 195,000-M_r peptide of fragment A into the 70,000-M_r peptide upon photocleavage (Fig. 6 *C*). Epitope 1 remains in the 130,000-M_r peptide of fragment A as described above. From the location of epitope 2 and of the 8-N$_3$ATP–binding site in photocleaved fragment A, we deduce that the 125,000- and 70,000-M_r photocleavage peptides represent the amino- and carboxy-terminal portions, respectively, of the original 195,000-M_r peptide (Fig. 7).

Preliminary experiments involving the irradiation of preformed fragment A in the presence of 0.1 mM V_i and 1 mM Mn^{2+}, in the absence of ATP, indicate that cleavage at the V2 site cuts the 195,000-M_r peptide of fragment A

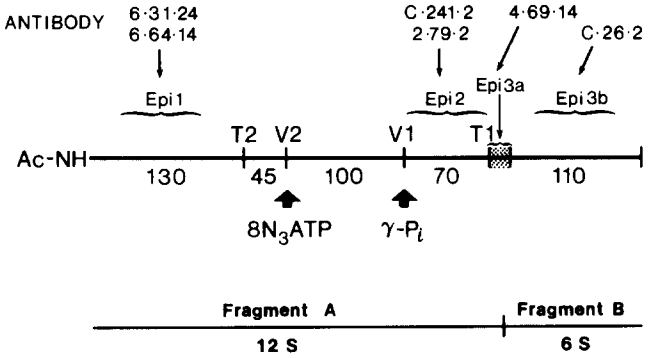

Figure 7. Linear map of the β heavy chain. Numbers below the line represent molecular mass in thousands, determined by electrophoretic migration in gels. The sites of V_i-mediated photocleavage are indicated as *V1* and *V2*. The principal sites of tryptic cleavage are indicated as *T1* and *T2*. The shaded section of the line represents the region of ~20,000 D that is lost in the formation of fragment B; its position shown is arbitrary for it is not known whether it is removed from the amino- or carboxy-terminal end of the 132,000-M_r peptide formed by the initial cleavage at the T1 site. Note that the summed value for the M_r of the intact heavy chain is 475,000, rather than 428,000 as reported earlier (see Discussion). The site of V1 photocleavage is believed to be associated with the γ-P_i binding region of the nucleotide-binding site (10). The site of V2 photocleavage is thought to be close to the site of 8-N$_3$ATP attachment (36).

into peptides of ~150,000 and 45,000 M_r. The molecular masses of these photocleavage peptides support the map of cleavage sites deduced above (Fig. 7).

Discussion

Prevous evidence has indicated that the sites of V1 and V2 photocleavage lie in the midregion of the β chain, separated by a distance of ~100,000 D on two separate loops of the tertiary structure that constitute part of the nucleotide-binding site. The former appears to be associated with the γ-P_i–binding region, and the latter with the group of residues to which the activated nitrene of 8-N$_3$ATP attaches (10, 36). The present data showing that all the epitopes for which we have antibodies are located toward either the amino or the carboxy regions of the β chain, outside the central portion that includes the two loci of the ATP-binding site, are consistent with the hypothesis that this central portion corresponds to the more functionally active, more evolutionarily conserved, and therefore less antigenic, region of the chain. The appearance of multiple clones of antigen against the same epitopes in the region between the amino terminus and the T2 site, as well as in the region between the V1 and T1 sites (Fig. 7), suggests that these regions are more active antigenically, possibly because they correspond to structural, rather than functional, regions of the chain that have been less conserved during evolution. The evolutionary variability of these regions of the dynein heavy chains is also emphasized by the fact that none of the monoclonal antibodies obtained against the heavy chains of *Chlamydomonas* dynein cross-reacts with sea urchin dynein, although an antibody against one of the intermediate chains does cross-react (15).

The relative functional roles of the α and β heavy chains in producing sliding between tubules remains unknown. The

two chains appear to have a similar, although not identical, electron microscopic structure (29). In addition, the chemical structure in the vicinity of their ATP binding sites is similar in that they both show V_i-mediated photocleavage at V1 and V2 sites at nearly the same position on the length of the two chains (10, 36). Although the isolated α and β chains have very different patterns of trypsin digestion, with the β chain forming a small number of semi-stable tryptic peptides corresponding to the fragments A and B considered here, and the α chain being cleaved more rapidly at multiple sites to form numerous heterogeneous peptides (21), this seems likely to be a result of partial denaturation of the α chain during the low salt separation procedure (1) rather than to a major difference in the tertiary structure of the heavy chains in native dynein. This partial denaturation of the separated α chain is evidenced by the marked decrease in its specific ATPase activity (35) and by the fact that it is no longer susceptible to V_i-mediated photocleavage (16). The greater stability of the β heavy chain during isolation may be due to an inherently greater stability in a low salt medium or to its tight association with intermediate chain 1 (35). This difference in stability of the two heavy chains cannot be taken as evidence of a substantial dissimilarity in their basic tertiary structure, such as would be indicative of a major difference in function.

As mentioned in the introduction, the molecular mass of the intact β heavy chain can be obtained by summation of its component photolytic and proteolytic peptides. However, the value obtained appears to depend upon the choice of peptides used. Summation of the HUV1 and LUV1 or of the HUV2 and LUV2 photolytic peptides results in a total M_r of 430,000 as reported previously (16, 36). Summation of the tryptic peptides shown in Fig. 4 of this paper leads to a total M_r of 455,000. Summation of the smallest peptides produced by consecutive photolytic and proteolytic cleavage, as shown in Fig. 7, results in a total M_r of 475,000. In part, these discrepancies may be the result of an anomalous electrophoretic migration of one or more of the dynein cleavage peptides (36). However, it seems likely that the major part is due to an underlying discrepancy in the standard proteins used to calibrate the electrophoresis gel. The heavy chain of skeletal muscle myosin and the β chain of spectrin have reported molecular masses of 224,000 and 220,000, respectively, with the value for myosin being based upon complete amino acid sequence and that for spectrin upon peptide summation (31, 32). However, the β spectrin chain migrates significantly more slowly upon electrophoresis gels (e.g., Figs. 2 and 5 in reference 36). It is not yet known whether this is due to anomalous migration or whether the reported M_r of the α and β spectrin chains is an underestimate. Be this as it may, we note the best internal agreement for the various dynein peptides described here is obtained by assigning the β heavy chain of sea urchin dynein an M_r in the vicinity of 475,000, with a comparable upward adjustment of the reported values for its V1 and V2 cleavage peptides. Exact values will hopefully be revealed by gene cloning in the near future.

The structure of the dynein β heavy chain may be compared with that of the myosin heavy chain. The distance between the putative sites of 8-N_3ATP attachment and of γ-P_i binding is ~100,000 D in the dynein heavy chain (Fig. 7), with the photoaffinity probe attaching at the NH_2-terminal end of this region (36). Likewise for myosin, there are indications that a substantial length of the heavy chain may be involved in forming the ATP-binding site. In this case, the region involved in purine binding lies toward the NH_2-terminal end of the chain around Trp-130 (20), whereas the SH1 and SH2 cysteines, whose cross-linking results in the trapping of ATP on the hydrolytic site (40), are located toward the carboxy end of the globular head region of the heavy chain at approximate positions 705 and 695, respectively (32). The substantial difference between the nucleotide specificities of dynein and of myosin (6), together with the fact that myosin is not subject to photocleavage when irradiated in the presence of low micromolar concentrations of V_i and ATP, makes it seem unlikely that there is a close homology between the structures of their ATP-binding sites. However, the purine-binding region lies toward the NH_2-terminal end of the binding site in both cases.

The striking difference between the electron microscopic structures of dynein and of myosin is accompanied by a difference between the position of the ATP-binding region on the heavy chains, which is located at the NH_2-terminal region of the heavy chain in myosin and in the midregion of the heavy chain in dynein. This midchain location of the binding site in dynein may be a reflection of its more complex role in cross-bridge function, possibly related to the apparently stable connections of the arms to both the A and B tubules that are visible in rapidly frozen flagellar axonemes (11, 29), and that may provide some of the resistive components required to convert tubule sliding into flagellar bending. The finding that brief tryptic cleavage of dynein arms bound to flagellar tubules severs the connection between enzymatic and structural domains of the arms so that the former become solubilized from their attachment sites on the B tubules upon addition of ATP (2, 33, 34) is consistent with tryptic fragment A of the β chain constituting one of the enzymatic domains of the outer arm.

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