Chymotryptic Digestion of Tetrahymena 22S Dynein.
I. Decomposition of Three-headed 22S Dynein to One- and Two-headed Particles

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Abstract. Molecular composition of Tetrahymena ciliary dynein has been examined by electron microscopy and gel electrophoresis. SDS-urea gel electrophoresis revealed that Tetrahymena 22S dynein contains three (Aa, Ab, and Ac) heavy chains whereas 14S dynein contains only one. The molecular masses of 22S and 14S dynein heavy chains were estimated to be ~490 and 460 kD, respectively. Electron microscopy of negatively stained specimens showed 22S dynein has three globular heads and thin stalks, whereas 14S dynein consists of a single head.

Chymotrypsin digested each of the three 22S dynein heavy chains into large fragments with different time courses. Sucrose density gradient centrifugation separated the digestion products as two peaks. The one with a larger sedimentation coefficient mainly consisted of two-headed particles having binding ability to doublet microtubules, whereas the other with a smaller sedimentation coefficient consisted of only isolated globular particles. Both fractions had ATPase activities. Thus, one active head of 22S dynein can be isolated by chymotrypsin digestion.

Dynein and microtubules constitute an important motile system in eukaryotic cells. In cilia and flagella, two rows of dynein reside on doublet microtubules and make crossbridges with adjacent doublet microtubules. Although the basic mechanism of movement is the sliding between adjacent doublets, and is thus similar to that in the actin–myosin system, dynein differs from myosin in both morphological and biochemical properties. Tetrahymena cilia contain two classes of dynein termed 30S and 14S dynein by Gibbons and Rowe (10). However, recent measurements of sedimentation coefficients have provided the value of 21S or 22S for "30S" dynein (15, 20). The term "22S (21S) dynein" is now widely accepted, and will be used in this paper. Recent structural studies on isolated dynein molecules have revealed that a dynein with ~20S from several sources has multiple heads and stalks (11, 15, 27, 31, 35). By analysis of 1.9 million for an integral molecule.

In this study, electron microscopy of 22S dynein negatively stained at a more neutral pH than formerly used was performed and confirmed the three-headed flower-bouquet structure. Further, SDS-urea gel electrophoresis revealed that 22S dynein comprises three heavy chains (HCs) of ~490 kD with different immunogenicity.

To examine the function of each head and its relationship to HCs, it is desirable to isolate these HCs. Since Tetrahymena 22S dynein is stable in high and low salt in contrast to sea urchin 21S dynein (29, 36) and Chlamydomonas 18S dynein (23), proteolytic digestion was attempted to isolate each head. In this paper, I describe the decomposition of 22S dynein into two functional fragments by chymotryptic digestion. A detailed analysis of the degradation pathways of the HCs will be described in the following paper (32).

Materials and Methods

Preparation of 22S Dynein

Tetrahymena pyriformis (strain W) and Tetrahymena thermophila (strain B-255, gift from Dr. E. Orias, University of California, Santa Barbara, CA) were grown in a medium containing 1% protease peptone, 0.5% yeast extract (both from Difco Laboratories, Inc., Detroit, MI), 0.87% glucose, and a small amount of anti-foaming agent (Nakarai Chemical Co., Kyoto, Japan) with aeration at 25°C. After collecting cells by low-speed centrifugation, cilia were isolated and demembranated as described by Porter and Johnson (25). The isolated axonemes were resuspended in 0.6 M NaCl, 10 mM Hepes, pH 7.4, 4 mM MgCl2, 0.1 mM EGTA, 1 mM diithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), at ~10 mg/ml, left to stand for 20 min at 0°C, and centrifuged at 40,000 g for 20 min.

The extract was fractionated on a 16-ml of 5-25% (wt/wt) sucrose density gradient in a solution of 0.1 M NaCl, 4 mM MgCl2, 0.1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, and 10 mM Hepes, pH 7.4 (H buffer). After the sedimentation at 26,000 rpm for 19-20 h at 4°C in a model No. RPS 27-2 rotor of an ultracentrifuge (model 55P; Hitachi Ltd., Tokyo, Japan), the content of the centrifuge tube was separated into 20 or 22 fractions of equal volume. The fractions of the first and the second peaks of protein with ATPase activity were pooled and used as 22S and 14S dynein preparations, respectively.

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1. Abbreviations used in this paper: HC, heavy chain; STEM, scanning transmission electron microscope.
Chymotryptic Digestion of 22S Dynein

The 22S dynein preparation was exhaustively dialyzed against the H buffer to remove sucrose. Protein concentration was adjusted to 0.5 mg/ml. Amicon XM 100A ultrafiltration membrane was used to concentrate specimens as necessary. Digestion was started by adding α-chymotrypsin (Sigma Chemical Co., St. Louis, MO) to 0.1 mg/ml at 25°C, and terminated by adding PMSF to 1 mM.

The digest was fractionated on a 5–20% (wt/wt) sucrose density gradient in the same buffer. The centrifugation and fractionation were done as in the preparation of 22S dynein.

For the markers of sedimentation coefficient, phosphorylase b (8.8S), catalase (11S), β-galactosidase (16.1S), and thyroglobulin (19.2S) (all from Sigma Chemical Co.) were used.

To fractionate the digest by ion exchange column chromatography, DEAE-Sephacel (Pharmacia, Uppsala, Sweden) equilibrated with the H buffer was used and eluted with a gradient of NaCl from 0 to 0.6 M.

Measurement of Binding Ability to Doublet Microtubules

To obtain doublet microtubules, axonemes were washed with 0.1 mM EDTA, 0.1 mM DTT, and 1 mM Tris-HCl, pH 8.0, after high salt extraction, and dialyzed against the same buffer for 2 d. The doublet microtubules were collected by centrifugation at 30,000 g for 20 min and suspended in the H buffer. Dynein and microtubules were mixed to the final concentrations of 0.15 and 0.60 mg/ml, respectively. After incubation for 20 min at 0°C, the mixture was centrifuged at 50,000 g for 15 min. The supernatant and the pellet were subjected to electrophoresis. The effect of ATP was examined by adding ATP to 0.1 mM just before centrifugation.

Measurement of ATPase Activity

For the assay of ATPase activity, a sample was added to 1 ml of an assay medium containing 0.1 M NaCl, 4 mM MgCl₂, 1 mM ATP, and 10 mM Tris-HCl, pH 7.5, and incubated for 10 min at 25°C. The liberated inorganic phosphate was measured as described by Murphy and Riley (21).

Determination of Protein Concentration

Protein concentration was determined at first by measuring the absorbance at 280 nm using an extinction coefficient of 1.0 cm²/mg. However, unknown materials, which contributed to the absorbance at 280 nm but not to the dye-binding assay, usually existed in the dynein preparation. Thus, the Bradford method (5, 26) was used.

PAGE

SDS-PAGE using a discontinuous Tris-glycine buffer system (16) and a continuous phosphate buffer system (34), and SDS-urea-PAGE were performed as described previously (36), except that 3.2% slab gels were used for SDS-urea gel electrophoresis.

Native gel electrophoresis (8) was carried out on 3% polyacrylamide slab and disc gels.

Electron Microscopy

One drop of specimen solution containing 20–50 μg/ml of proteins was applied to a very thin carbon film supported by a carbon-perforated film, and stained with 1% uranyl acetate (unbuffered) or 1% uranyl oxalate (pH 6.0) (12). Specimens were examined in a JEOL JEM 100CX operated at 80 kV, Hitachi H700 at 100 kV at the nominal magnification of 50,000×. Magnification was calibrated by measuring the 39.5-nm striation of tropomyosin tactoids (6).

Results

Heavy Chains of Tetrahymena 22S and 14S Dynein

High-salt extracts of axonemes from Tetrahymena form two peaks on a sucrose density gradient; they have been termed 22S (15) and MS (10) dynein. Fig. 1 a shows the SDS gel electrophoresis pattern of 22S and 14S dynein preparations on

![Figure 1](image_url)
a 6% slab gel in a discontinuous Tris–glycine buffer system (16). The 22S fraction (lanes 3 and 5) showed a major dense band of high molecular mass (heavy chain [HC]) and faint bands about halfway down the gel (intermediate chains). The gel of the 14S fraction (lanes 4 and 6) showed an HC band with slightly higher mobility than that of the 22S fraction, several intermediate chains, and a contamination of tubulin. HCs of 22S and 14S dynein fractions have been termed A-band and B-band dynein, respectively (18).

Fig. 1 b shows the SDS gel electrophoresis pattern on 3% gels containing 6 M urea in a continuous Tris–glycine buffer system (36). HCs of 22S dynein (lanes 3 and 5) appeared as three bands with apparently equal density through the fractions of the 22S peak. These three HC bands will be termed Aa, Ab, and Aa in the ascending order of mobility. The masses of these HCs were estimated by electrophoresis in a phosphate buffer system (34) as 490 kD (lanes 3 and 5); the mass of 14S dynein HC was ~460 kD (lanes 4 and 6). The mobilities of 22S dynein HCs in these three buffer systems were very similar to those of A-band HCs of 21S dynein from sea urchin sperm flagella (lane 1).

Electron Microscopy of 22S and 14S Dynein

Fig. 2 shows large fields of view of 22S and 14S dynein preparations which were stained with uranyl oxalate, pH 6.0, to avoid possible artefacts from acidic staining reagents. In accordance with Johnson and Wall (15) and Goodenough and Heuser (11), the 22S dynein molecule was seen to have three globular heads and three stalks.

These three globular heads were similar to each other in both shape and size (~16 × 14 nm) and had a stain accumulation around the center. From the views tilted by 40° and 55°, the ellipticity of the head was estimated to be 0.59 (SD = 0.05, n = 29) (7, 30). Thus the rough estimate of the volume of one head was 1,050 nm³, which gave the mass of 460 kD assuming a partial specific volume of 0.73.

Two stalks are bound together near their midpoint, where an additional mass appears as a small blob, and where these two stalks are seen to connect to the third one. The third stalk looks longer and thicker than the others due to another extra mass, which occasionally projects out from the stalk to form a “rootstock” (arrowhead in Fig. 2 a; also see Fig. 2 of Johnson and Wall [15]). These features agree well with the rapid-freeze rotary-shadowed images by Goodenough and Heuser (11), especially in the asymmetry of the molecule. The reported value for the dimension of the head (φ = 13.2 nm) is slightly smaller than the value obtained here.

Images of the 14S dynein preparation showed that the particles were isolated and had stain accumulation around the center (Fig. 2 b). The diameter (18 nm) was larger than that of one head of 22S dynein. Some particles appeared C-shaped (indicated by arrows in Fig. 2 b). It is not clear whether 14S dynein also has a stalk.

All of the micrographs presented here and in Johnson and Wall (15) were obtained with the preparations from a mutant T. thermophila B 255. 22S dynein from T. pyriformis W was also examined by electrophoresis (Fig. 1) and electron microscopy. Since there was no difference between these two species, T. thermophila B 255 was exclusively used for further analyses.

Chymotryptic Digestion of 22S Dynein

Since changes in ionic strength were ineffective for decomposing the Tetrahymena 22S dynein, three proteases (namely, trypsin, chymotrypsin, and V8 protease from Staphylococcus aureus) were tried. Chymotrypsin produced large fragments which entered into the matrix of 3% polyacrylamide gels under nondenaturing conditions (Fig. 3 a); trypsin digested too extensively while V8 protease cleaved too slightly.

In the course of chymotrypsin digestion, six major bands with high molecular masses (490–320 kD) were resolved on SDS–urea gels and were numbered as shown in Fig. 3 b. Note that Aa, Ab, and Aa HCs correspond to band-1, -2, and -3 HCs, respectively. It must be emphasized that actually three HC bands (3a–3c in the ascending order of mobility in the Laemmli system) with different origins contributed to this band, although band-3 HC apparently persisted, as described in the following paper (32).

The mass of each of these HC fragments was estimated by SDS gel electrophoresis in phosphate buffer system (34) as 430 kD (band 3b), 410 kD (band 3c), 370 kD (bands 4 and 5), and 320 kD (band 6), respectively.

It is convenient to divide the digestion process into three stages (1–III), although stage III could not be reached by prolonged digestion with small amounts of protease. Also the production of band-6 HC appeared to depend on the amount of chymotrypsin. These stages are characterized by the disappearance of band-1 HC (I), appearance of band-4 HC (II), and disappearance of band-2 HC (III). Mg-ATPase activity increased twofold during stages I and II and was constant in stage III (Fig. 4).

Fractionation of Digestion Products

Native gel electrophoresis (Fig. 3 a) showed the production of large isolated fragments by chymotryptic digestion. They formed two (H and L) peaks on a sucrose density gradient, and both had ATPase activities (Fig. 5). H peak, ~18S, consisted of band-2 and -5 HCs at stage I; band-2, -3b, and -5 HC at stage II; band-3b, -5 and -6 HCs at stage III. L peak, ~11S, consisted of band-3c HC at stage I; band-3c and -4 HCs at stage II; and band-4 HC at stage III (Fig. 6), taking the identification of band-3 HCs into account (see Fig. 2 of reference 32). However, the composition of polypeptides varied within fractions of H peak: at stage I, for instance, band-2 and -5 HCs were major components in the fractions with larger S values whereas band-2, -3, and -5 HCs were present equally in those with smaller S values (Fig. 5).

When digested very briefly, there was another peak in addition to H and L peaks at slightly smaller than 22S. This new peak consisted of band-1, -2, and -5 HCs; H and L peaks had compositions similar to those at stage I, although several other HC fragments, which appeared to be digested further rapidly, were also present (Fig. 7). Since the particles in this new peak apparently kept integrity of the molecule, the digestion of band-1 (Aa) HC seems to be responsible for the decomposition of 22S dynein to H and L fragments.
Figure 3. "Native gels" (a) and SDS-urea gels (b) showing the time course of chymotryptic digestion of 22S dynein. 22S dynein (0.5 mg/ml) was digested with α-chymotrypsin (0.1 mg/ml) for 1–60 min at 25°C. HCs and their major fragments are numbered in b. Roman numerals at the top indicate the digestion stages (see text for the definition); Arabic numbers show the digestion time.

Figure 4. Effect of chymotryptic digestion on ATPase activity of 22S dynein. 22S dynein (0.5 mg/ml) was digested with α-chymotrypsin (0.1 mg/ml, solid circles; 0.05 mg/ml, open circles) for 1–90 min at 25°C. Note that stage III could not be reached with 0.05 mg/ml α-chymotrypsin. Ordinate, ATPase activity relative to that of intact 22S dynein; abscissa, digestion time.

Discussion

Molecular Mass of 22S Dynein Heavy Chain

The molecular mass of dynein HC has been estimated by many workers by SDS gel electrophoresis (see reference 1), and values ranging from ~350 (25, 33) to 600 kD (19) have been reported. This large discrepancy may have originated from differences in buffer systems and molecular mass markers. To get good linearity and to avoid extrapolation, we used a phosphate buffer system (34) and appropriate acrylamide concentration. The values (500 kD) for sea urchin 21S dynein HCs obtained with this system (36) agree well with the

Binding of Digestion Products to Doublet Microtubules

In the absence of ATP, 22S dynein mixed with doublet microtubules were all sedimented by ultracentrifugation (Fig. 9 a, -ATP). By the addition of ATP, about one-half was dissociated (Fig. 9 a, +ATP), showing that 22S dynein binds to microtubules in both an ATP-sensitive and an ATP-insensitive manner (28). When the digest at stage II was mixed with doublet microtubules, band-2, -3b, and -5 HCs bound to microtubules but band-4 HC did not; ATP dissociated these bound HCs (Fig. 9 b), indicating that the binding was entirely ATP sensitive. Fig. 9, c and d shows that the particles in H peak bound to doublet microtubules in an ATP-sensitive manner (c) whereas those in L peak did not (d). The same results were obtained with the digest at stage III.
values (430–500 kD) obtained by sedimentation equilibrium measurement (2) and by UV cleavage of HCs (17).

STEM mass measurement provided the value of 1,950 kD for an integral molecule and 400–550 kD for each globular head (15). Considering that 22S dynein consists of three HCs and several intermediate chains, these values give 500–600 kD for the mass of one HC, which agrees well with the present estimate (490 kD).

Figure 6. SDS–urea gel electrophoresis of the digestion products fractionated by sucrose density gradient centrifugation. H and L refer to H peak and L peak on a sucrose density gradient (see Fig. 5); 22S, 22S dynein before digestion; I–III, digestion stages; other numbers show the identification of HC bands.
Figure 8. Electron micrographs of peak H (a) and L (b) fractions of the digestion products of 22S dynein. Negatively stained with 1% uranyl oxalate, pH 6.0. Bar, 50 nm.
**Subunit Structure of 22S Dynein**

SDS–urea gel electrophoresis resolved three HCs in the 22S dynein preparation (Fig. 1b). As described in the following paper, these three HCs of 22S dynein have different immunogenicity. Thus, the number of distinct HCs corresponds to the number of heads for *Tetrahymena* 22S dynein, as is the case for sea urchin 21S dynein, *Chlamydomonas* 18S and 12S dynein and starfish 20S dynein: sea urchin 21S dynein consists of two HCs (4, 36) and two heads (27, 31); *Chlamydomonas* 18S dynein has two HCs (14) and two heads (35); while 12S dynein has a single HC (14) and a single head (35); starfish 20S dynein consists of two HCs (19) and two heads (31).

**Chymotryptic Digestion of 22S Dynein**

Chymotryptic digestion of 22S dynein, providing the means to isolate at least one of three heads in an almost intact form (L fragment), is very useful for studying the submolecular structure of dynein. This band-4 HC may correspond to the head at the left end in the inset of Fig. 2a, because the tail of the particles found in peak H extended beyond the junction point and the distance from the head to junction point was short (Fig. 8a). Since the cleavage of band-1 HC resulted in a decomposition of the molecule and loss of ATP-insensitive binding, a portion of band-1 HC (>80 kD), probably at the "base" of the molecule, seems to be vital for maintaining the integrity of the molecule and the ATP-insensitive binding site. Although it is not known which two of the three heads of *Tetrahymena* 22S dynein corresponds to the two heads of sea urchin 21S dynein, it is interesting that sea urchin 21S dynein also loses ATP-insensitive binding ability when Aa HC is degraded by proteolysis (3).

Chymotrypsin decomposed a three-headed 22S dynein into a two-headed H fragment and a single globular L fragment, both associated with ATPase activity. However, unless we determine the degradation pathway, we cannot conclude that there are multiple ATPase sites in 22S dynein, since L fragment may contain the digestion product of H fragment. This point is discussed further in the accompanying paper (32). In the course of digestion, ATPase activity increased more than twofold, as also reported by Hoshino (13) for trypptic digestion. This result supports the idea of "latency" for intact dynein ATPase (9).

Goodenough and Heuser (11) showed that *Chlamydomonas* dynein is also three-headed and can be decomposed to two-headed 18S and one-headed 12S dynein, and that two-headed...
and single-headed particles are also found in Tetrahymena dynein preparations. Chlamydomonas 18S and 12S dynein also have ATPase activity (24). The relationship of H and L fragments to these subunits is the subject of future studies.

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