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ATPase sites in two-headed fragment of *Tetrahymena* 22S ciliary dynein

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Three-headed *Tetrahymena* 22S ciliary dynein was found to consist of three heavy chains (HCs) and decompose into two-headed and single-headed fragments upon chymotrypsin digestion. The three HCs (Aα, Aβ, and Aγ) were immunologically different, and presumed to be located on each of the head regions. The two-headed fragment contained Aβ and Aγ HCs, while the Aα HC originated in the single-headed fragment. Both fragments were associated with ATPase activity (Toyoshima, Y. (1987a) J. Cell Biol. 105, 887–895 and Toyoshima, Y. (1987b) J. Cell Biol. 105, 897–901). Using the two-headed dynein fragment, we attempted to determine the site of ATP hydrolysis in the fragment. After digestion of the fragment with 100 μg/ml thermolysin for 45 min, we noted eight thermolysin-digested polypeptides (TH 1, 2, 3, 4, 5α, 5β, 6α, and 6β). By precisely analyzing the degradation process and the products using peptide mapping, immunoblotting and high pressure liquid chromatography, it appeared that the two-headed fragment is dissociated as two separate fragments, each of which contained Aβ or Aγ HC. Thermolysin digests, TH 1, 2, 5α and 6β were found to be derived from Aβ HC, while TH 3, 4, 5β and 6α originated in the Aγ HC. Based on the measurements of ATPase activity of these polypeptides, we concluded that the ATPase site is located in the Aβ and Aγ HCs, which may have their origins in each head of the two-headed fragment of *Tetrahymena* 22S ciliary dynein.

Introduction

It has been established that the bending movement of cilia and flagella is brought about by a sliding of the adjacent doublet microtubules in the axonemes [3]. Dynein arms projecting from the A-tubule of the doublet microtubules are supposed to bind with the cross-bridge and push the B-tubules of the adjacent doublet microtubules into sliding along the longitudinal axis, hydrolysing ATP [4]. Although several characteristics of this sliding movement between the doublet microtubules have been analyzed [5–7], little is known about how the dynein arm induces the sliding movement and transforms the chemical energy of ATP into mechanical displacement. To elucidate the mechanism of such movement, the submolecular structure and morphological characteristics of dynein molecules should be studied.

Yano and Miki-Noumura [8], observing negatively stained 21S outer dynein arms isolated from sea urchin sperm flagella, first reported the appearance of a Y-shaped structure having two globular heads. Sale et al. [9] visualized the 21S dynein to be composed of two globular heads joined by two stems, using a quick-freeze, deep-etch technique, and they were able to show two heavy chains, Aα and Aβ, in the head region. The outer arm dynein from *Chlamydomonas* flagella was composed of three discrete subunits, (α, β, and γ), each of which exhibited ATPase activity. The α and β subunits were purified as a heterodimer complex having a sedimentation coefficient of 18S, while the γ subunits were purified as a discrete particle that sediments at 13S. The 18S dynein consists of two heads connected by a Y-shaped stalk, in contrast to 13S dynein, which was a single globular unit [10,11].

The three-headed shape was observed in the isolated dynein of *Tetrahymena* cilia [12]. Based on observations by scanning transmission electron microscopy, Johnson and Wall [12] proposed a 'flower-bouquet' model of *Tetrahymena* ciliary dynein, which consisted of three globular heads connected by three separate stalks to a rootlike base. Mass analysis of the dynein molecule indicated a molecular weight of 2.17 ($\pm$0.14) megadaltons. After confirming the flower bouquet structure of *Tetrahymena* 22S ciliary dynein, Toyoshima [1] reported that the three-headed dynein of *Tetrahy-*

Abbreviations HC, heavy chain; CT, chymotrypsin digested.

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mena was composed of three heavy chains, Aα, Aβ and Aγ, each of which corresponded to one head. Chymotrypsin digestion decomposed the 22S dynein into two-headed and single-headed fragments, both of which are associated with ATPase activity. The two-headed fragment contained Aβ and Aγ HCs, whereas the Aα HC originated from the single headed-fragment [1].

Vale and Toyoshima [13], using a quantitative in vitro assay, showed that both 22S and 14S dyneins from *Tetrahymena* cilia induce movement with distinct motile properties. Furthermore, Vale and Toyoshima [14] have recently explored the function of the multiple ATPase heads of 22S dynein by using the single- and the two-headed proteolytic fragments from 22S dynein and examining their motile activity in vitro. Although the single-headed fragment did not induce movement, the two-headed fragment translocated microtubules at velocities similar to those of intact 22S dynein.

According to Tang et al. [15], the 21S dynein of sea urchin sperm flagella contained two distinct dynein ATPase, and each heavy chain, α and β of 21S dynein, had ATPase activity. However, Sale and Fox [16] reported recently that the actual functional ability of HCs is different, that is, microtubule gliding activity was coincident with the β-HC-intermediate chain 1 fraction, but not with the α-HC-intermediate-chains 2 and 3 fraction.

In the study presented here, we attempt to determine whether Aβ and Aγ HCs of the two-headed fragment of *Tetrahymena* ciliary dynein are associated with ATPase activity, by analyzing the thermolysin digests of the two-headed fragment, using peptide mapping, immunoblotting and fractionation with high pressure liquid chromatography.

Material and Methods

Preparation of dynein

Tetrahymena thermophila, strain B-255 was used. The cells were cultured in 1% PYD culture medium (1% proteose peptone, 0.5% yeast extract, 0.87% glucose) and a small amount of anti-foaming agent with aeration at 2°C. After collecting the cells with low-speed centrifugation, cilia were isolated with 5 mM dibucaine-HCl and demembranated, as described by Porter and Johnson [17]. The isolated axonemes were suspended in dynein extraction medium (0.6 M NaCl, 10 mM Hepes, 4 mM $MgCl_2$, 1 mm EGTA, 1 mM dithiothreitol (DTT), pH 7.4) for 30 min at 0°C. After centrifuging this suspension at 100000 × *g* for 10 min at 2°C, the crude dynein was obtained as supernatant. Based on the method by Toyoshima [1], the supernatant was centrifuged on 5–25% sucrose-density gradient at 100000 × *g* for 19 h at 2°C, and fractionated into about 20 tubes, of which volume was 0.65 ml per tube. After measuring absorbance at 280 nm and ATPase activity, the first peak fractions in profiles of protein concentration and ATPase activity were pooled and used as 22S dynein. The 22S dynein fraction was dialysed against Hepes buffer solution (10 mM Hepes, 0.1 M NaCl, 4 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, pH 7.4) for 4.5 h at 4°C, with three buffer changes.

Preparation of the two-headed dynein

The 22S dynein was digested with α-chymotrypsin (Sigma Chemicals St. Louis, MO), using the method of Toyoshima [1,2]. The 22S dynein having a protein concentration of 0.5 mg/ml, was digested with α-chymotrypson of 20 μg/ml for 10 min at 25°C, followed by addition of 1 mM phenylmethylsulfonyl fluoride (PMSF), to terminate the digestion. After a 5–20% sucrose-density gradient centrifugation for 19 h at 2°C, the digested specimen was fractionated into 0.65 ml per tube. Peak fractions in protein concentration were dialyzed against Hepes buffer (without EGTA) for 5 h at 4°C, with three buffer changes. The dialysed specimen was used as the two-headed dynein.

Digestion of the two-headed fragment with thermolysin

The specimen of two-headed dynein was adjusted to be 0.2 absorbance units at 280 nm, followed to be digested with thermolysin (Sigma Chemicals, St Louis MO) in the presence of 2 mM $CaCl_2$ at 25°C. The digestion was terminated at an appropriate time during the digestion period by addition of 5 mM EDTA.

Measurement of ATPase activity

The specimen of two-headed dynein, 50 μl, was incubated with ATP and 20 μl of an assay medium containing 0.1 M NaCl, 4 mM $MgCl_2$, 10 mM tris-HCl buffer (pH 7.5) for 10 min at 25°C. Quantitaive analysis of liberated inorganic phosphate was done by the method of Murphy and Riley [18].

PAGE

SDS-PAGE in a discontinuous Tris-glycine buffer system [19] and SDS-urea-PAGE in a continuous Tris-glycine buffer system [20], were done for 14 h at 8 mA. A silver staining kit (Daiichi Chemicals, Tokyo, Japan) was used for the gel staining with silver. For determination of molecular weight, HMW marker (Pharmacia Chemicals, Sweden) was applied to Laemmli system.

Measurement of protein concentration

Protein concentration was determined by the method of Read and Northcote [21], using Coomassie blue G for dye-binding.

Preparation of antibodies

Antibodies against 22S dynein and its chymotrypsin digest were previously prepared by Toyoshima [2]. We

used the antibodies (given by Dr. Toyoshima) in this experiment, to identify whether thermolysin digests originate in HCs, Aα, Aβ or Aγ.

Immunoblotting

The specimen of antigen was first run on SDS mini-slab gel electrophoresis in Laemmli system, based on the method by Towbin et al. [2], the gel pattern was transferred to a nitrocellulose sheet of a pore size of 0.1 μm (Schleicher & Schuell, Keene, NH), which was incubated with the antibodies. A horseradish peroxidase-conjugated goat IgG raised against rabbit IgG (Cappel Laboratories, Melvern, PA) was used as the second antibody. The sheet was further stained with 4-chloro-1-naphtol (Bio-Rad laboratories, Richmond, CA).

Peptide mapping

The specimen previously treated with N-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide (DACM), was applied on SDS-gel (Laemmli) electrophoresis as reported by Yano-Toyoshima [20]. Illuminating with ultraviolet light, gel bands containing dynein HCs were cut out as a gel slice. For accurate comparison of peptide mapping, the two gel slices to be compared were put into the same sample slot in stacking gel of the Laemmli system. 5 μg of thermolysin in sample buffer was put into each slot. After electrophoresis for 14 h at 8 mA, the gels were stained with silver staining kit (Daiichi Chemicals, Tokyo).

High pressure liquid chromatography

The digested specimen, 3–4 ml, was applied an anion-exchange chromatography column (Mono Q, Pharmacia Chemicals, Sweden) and eluted with 50 ml of 0–1 M NaCl gradient solution at a speed of 1 ml/min, the fractionate into 500 μl per each tube.

Results

Chymotrypsin digestion of 22S ciliary dynein

Confirmation of the chymotrypsin digestion of 22S ciliary dynein was first made using *Tetrahymena* [1]. The three-headed 22S dynein was digested with 20 μg/ml chymotrypsin for 10 min at 25°C, and centrifuged in a 5–20% sucrose-density gradient. Profiles of protein concentration and ATPase activity corresponded well with each other, as shown in Fig. 1C. One major peak (fraction 11) and two small peaks (fractions 7 and 17) can be seen at both trailing shoulders of the major peak in the profiles. Fig. 1a and b present the electrophoretic patterns of the fractions in the Laemmli and SDS-urea systems. The SDS-urea gel patterns (Fig. 1b) clearly show that fraction 11 contained two heavy chains and fraction 17 consisted mainly of the one heavy chain. The same sedimentation profiles and electrophoretic patterns have been previously reported by Toyoshima [1]. The results indicate that fraction 11 containing the two-headed fragment and fraction 17 contained the single-headed fragment. The heavy chains in fraction 11 which separated into two components in the SDS-urea gel, were designated as CT (chymotrypsin digested) 2 and 5, and the heavy chain in fraction 17, as CT 3. Further study was then carried out on the two-headed fragment consisting of CT 2 and 5.

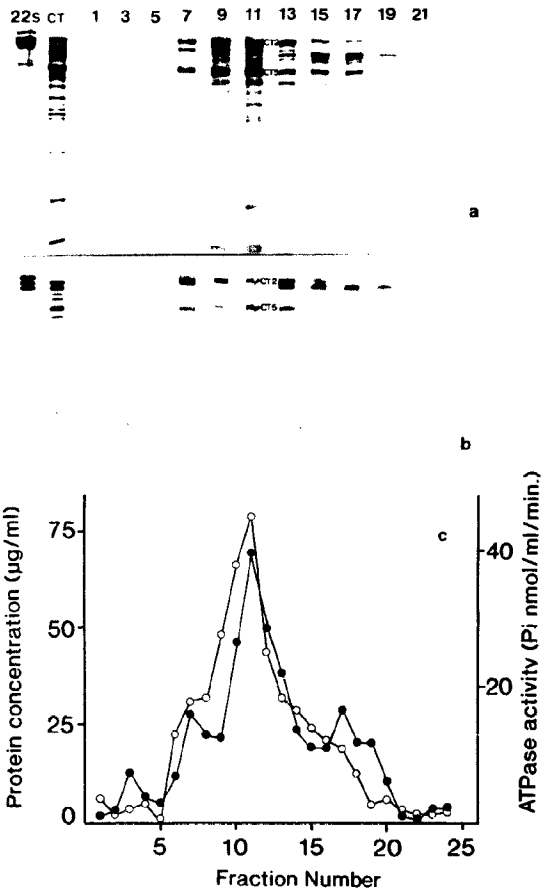

Fig. 1. Sucrose-density gradient centrifugation of chymotrypsin digest of 22S dynein. SDS-PAGE of 22S dynein (22S), the two headed-fragment (CT) and the fractions on a 6% polyacryamide gel in a discontinuous Tris-glycine buffer system (the Laemmli system) (a); SDS-urea-PAGE on a 3% polyacryamide gels containing 6 M urea in a continuous Tris-glycine buffer system (b). Numbers at the upper lane indicate the fraction number. Sedimentation profiles of protein concentration (●) and ATPase activity (○) (c). Ordinate: protein concentration (μg/ml) at the left; ATPase activity (P_i nmol ml per min) at the right. Abscissa: fraction number.

Major determinants of the mobility of polypeptides in gel electrophoresis are charge and size, that is, molecular weight of the polypeptide chains. The separation of protein by polyacrylamide electrophoresis is

dependent on the molecular weight of their polypeptide chains in the presence of the anionic detergents SDS. Based on the advantage of achieving thin starting zones by use of a discontinuous buffer solution, the SDS-discontinuous system, known as the Laemmli system, provides high resolution patterns of polypeptides. On the other hand, binding of SDS to the polypeptide chains changes in the presence of urea, depending on the charged state of the polypeptides. Therefore, two polypeptides in differently charged states having the same molecular weight are separated and migrated into two bands in SDS-urea system. Comparing the pattern in the SDS-urea system with that in the Laemmli system, three polypeptides in 22S dynein were clearly separated in the SDS-urea system (Figs. 1, 2 and 4), although their stained bands were a little broad.

Degradation process of two-headed dynein by thermolysin digestion

The two-headed fragment containing CT2 and 5 was dialyzed against Hepes buffer containing no EGTA for 4.5 h, with three buffer changes. We previously diluted the specimen to give 0.2 absorbance units at 280 nm. Digestion of the specimen was carried out at thermolysin concentrations of 20, 50, 75 and 100 μg/ml for 0 to 70 min at 25°C in the presence of 2 mM $CaCl_2$. The degradation process was analyzed by electrophoresis using the Laemmli and the SDS-urea systems. As shown in Fig. 2a and b, the thermolysin-digested products, TH 1, 2, 3, 4, 5(α, β) and 6(α, β) appeared and disappeared one after the other during the degradation process. Although separation was not so clearly defined in the SDS-urea system, the α and β bands in TH 5 and 6 clearly separated from each other in the Laemmli system. First TH 4 and then TH 1 and 6 (α, β) became visible in the gel pattern. Chymotrypsin digests, CT 2 and 5, were gradually digested and the stained bands became faint in appearance; this was simultaneously accompanied by the gradual appearance of TH 2, 4 and 5 (α, β) in the gel, TH 1 and 3 then disappeared. Comparing the stained bands in the SDS-urea system with those in the Laemmli system, and based on the molecular weight probe in the

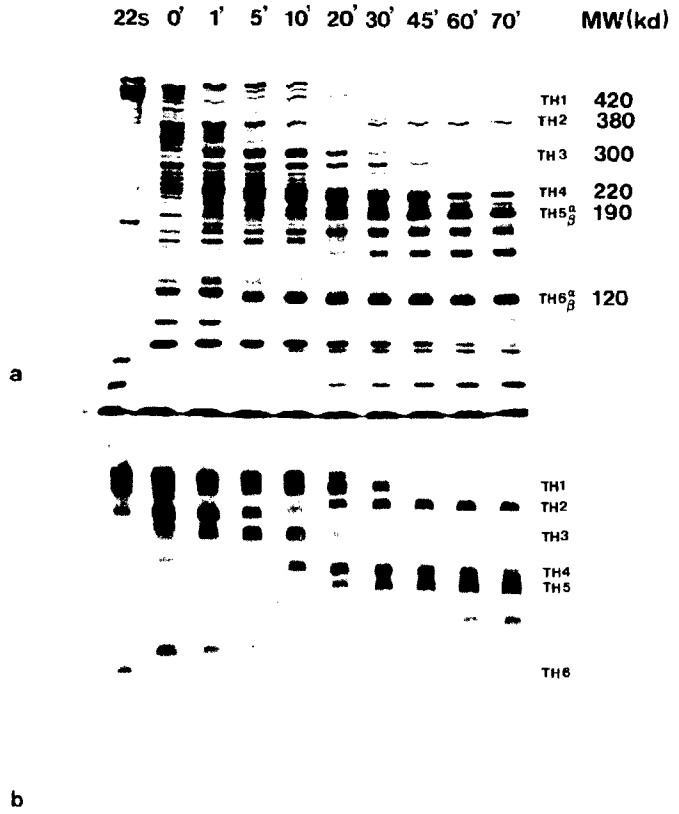

Fig. 2. SDS-PAGE (a) and SDS-urea-PAGE (b) showing the time-course of thermolysin digestion of the two-headed fragment. The two-headed fragment was digested with 100 μg/ml thermolysin for 0–70 min at 25°C. Digestion time is shown at the upper lane. The thermolysin digests, as TH 1–6, and the molecular weights, are shown at the right. PAGE of 22S dynein is shown at the left.

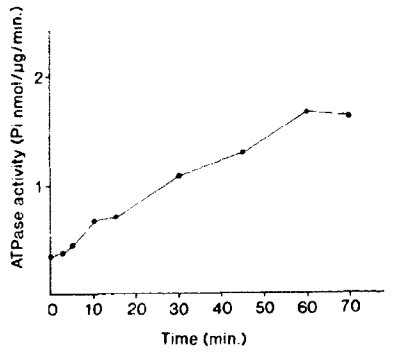

Fig. 3. Effect of thermolysin digestion on ATPase activity of the two-headed fragment, which was digested with 100 μg/ml thermolysin for 0–70 min. Ordinate: ATPase activity (P_i nmol/μg per min). Abscissa: digestion time (min).

Laemmli system, the molecular masses of the digested polypeptides of TH 1 to 6 were determined to be 420, 380, 300, 220, 190 and 120 kDa, respectively, and are indicated at the right side in Fig. 2.

The time-course for ATPase activity in the two-headed fragment during digestion with 100 μg/ml thermolysin was observed. Fig. 3 shows that ATPase activity increased gradually during digestion and reached a maximal level at 60 min. The ATPase activity increased to 4–5-times the level of that at the beginning of digestion.

Analysis of thermolysin-digested polypeptides using high pressure liquid chromatography

Polypeptides TH 2, 4, 5(α, β) and 6(α, β) were clearly visible as separate bands in the electrophoretic pattern produced using the Laemmli system, after CT 2 and 5 were digested with 100 μg/ml thermolysin for 45 min Fig. 2). To determine which TH polypeptide had ATPase activity, we first tried to separate the polypeptides using high pressure liquid chromatography. The thermolysin-digested specimens were applied to a Mono Q column for use with anion-exchange column chromatography and eluted with a 0 to 1.0 M NaCl gradient in Hepes buffer. Protein components

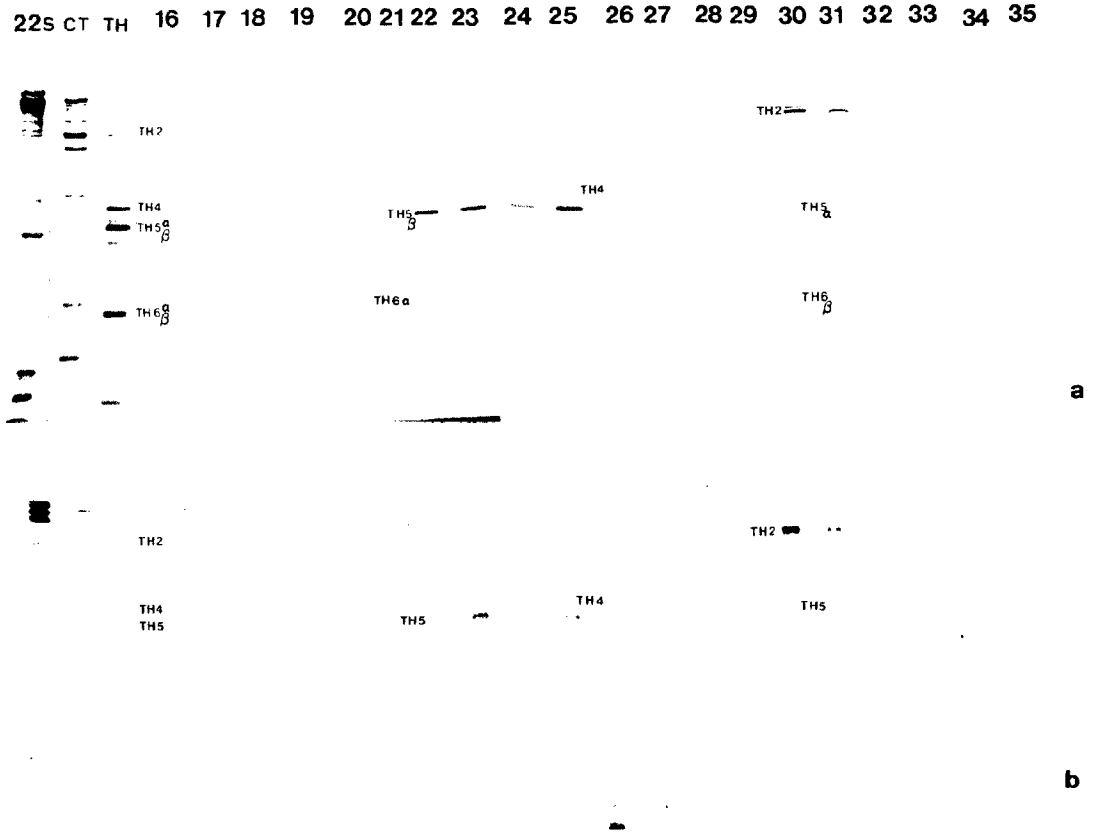

Fig. 4. SDS-PAGE (a) and SDS-urea-PAGE showing eluted fractions of thermolysin digests by high pressure liquid chromatography. Tube numbers of eluted fractions are shown at the upper lane. PAGE of 22S dynein (22S), the two-heated fragment (CT) and thermolysin digests (TH), are shown at the left.

were eluted with buffer solution containing 0.2 to 0.3 M NaCl, but no protein components were eluted with concentrations above 0.5 M NaCl. Electrophoretic patterns of fractions obtained from the SDS-urea and Laemmli systems are shown in Fig. 4. The patterns indicate a correspondence between eluted fractions and each thermolysin-digested polypeptide; TH5(β) polypeptide was observed with a faint TH (6(α), intermediate and lower bands were found in fractions 22 to 25, TH 4 together with TH 5(β) in fractions 26 to 28, TH 2 in fractions 30 to 31, and TH 2 together with TH 5(α) and 6(β) in fractions 30 to 33.

We next examined the ATPase activity of each fraction. First we noted a peak in the profiles of protein concentrations in fraction 19, which had no ATPase activity, although the protein component in fraction 19 was not analyzed yet. Peaks in the profiles of protein concentrations and ATPase activity were found in fractions 23, 25 and 31, as shown in Fig. 5. Although fractions 26 to 28 contained TH 4 and did not have a peak in the profiles, fraction 28, containing TH 4, was found to show a considerable amount of specific ATPase activity, that is, 5–6 P_i μmol/mg per min per 1 mM ATP. Fraction 30, containing TH 2, showed comparatively lower ATPase activity, whereas fraction 31 containing TH 2, 5(α) and 6(β), had considerable activity. The profile of ATPase activity and the gel patterns show that fractions 23 and 24 containing TH 5(β) with faint TH 6(α) and intermediate bands, have ATPase activity, while fraction 31 containing TH 2, 5(α) and 6(β) also has ATPase activity. Thus, two groups of thermolysin-digested polypeptides were found to have ATPase activity: one group consists of TH 2, 5(α), and 6(β), and the other group consists of

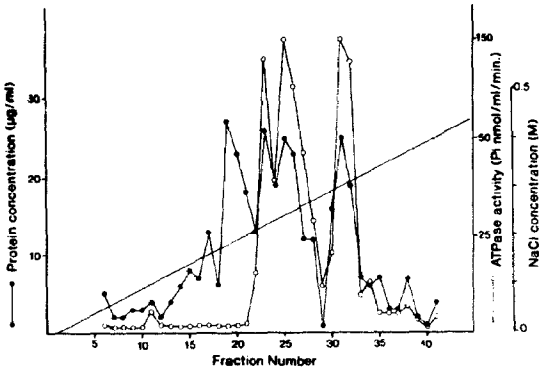

Fig. 5. Profile of ATPase activity (○) and protein concentration (●) of thermolysin digests (100 μg/ml thermolysin for 45 min) by high pressure liquid chromatography (Mono Q column). The solid line indicates the salt concentrations during the elution. Ordinate: protein concentration (μg/ml) at the left; ATPase activity (P_i nmol/ml per min) and NaCl concentrations at the right. Abscissa: fraction number.

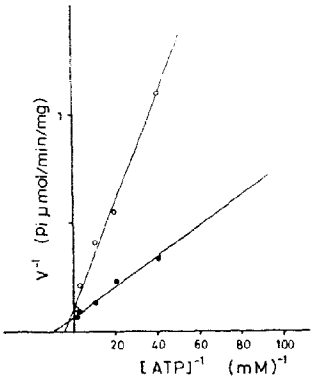

Fig. 6. Double reciprocal plots of ATPase activities of two fractions containing TH 2 (○) and TH 4 (●) at various concentrations of ATP. Ordinate: V^{-1} (P_i μmol/min per mg)$^{-1}$. Abscissa: S^{-1} (mM)$^{-1}$.

TH 4, 5(β), 6(α), intermediate and lower bands. Considering these findings along with an estimation of the molecular weights of the digests (Fig. 2), we suggest that TH 2 degradates into TH 5(α) and 6(β), while TH 4 degradates into TH 5(β), a faint TH 6(α) and digests of intermediate and lower molecular weight.

Whether or not the ATPase activity of TH 2 is really lower cannot be definitely determined at this time. We attempted to measure the ATPase activity of the fraction containing TH 2. A time-course showed that the ATPase activity of TH 2 was not proportional to the incubation time and decreased gradually with time. The lower rate is presumed at present to be due to inhibition by the product or the inactivation of ATPase with time. ATPase activity of two fractions containing TH 2 or TH 4, was first surveyed during shorter incubation times. Fig. 6 shows the double reciprocal plot of the data. The apparent Michaelis constant (K_m) appeared to be 100 μM for TH 4, and 250 μM for TH 2. The K_m values suggest that TH 2 and TH 4 have different characteristics in enzymic activity and they are perhaps two ATPases having different K_m values. At present further study on this point is in progress using purified TH 2 and TH 4.

Origin of thermolysin-digested polypeptides in CT 2 and 5

Immunoblotting. Polyclonal antibodies raised against CT 2 and 5 [2] were used. Antigens CT 2 and 5, and thermolysin digests which were digested with 100 μg/ml thermolysin for 5 or 45 min, were applied to a 5% mini-slab gel using the Laemmli system. Bands in the gel were transferred to a nitrocellulose membrane which was incubated in a solution containing the antibodies against CT 2 and 5. Fig. 7 shows the binding reaction between the antigens and the antibodies. The antibody against CT 2 reacted with antigens of CT 2, TH 1, 2, 5 and 6, while the antibody against CT 5

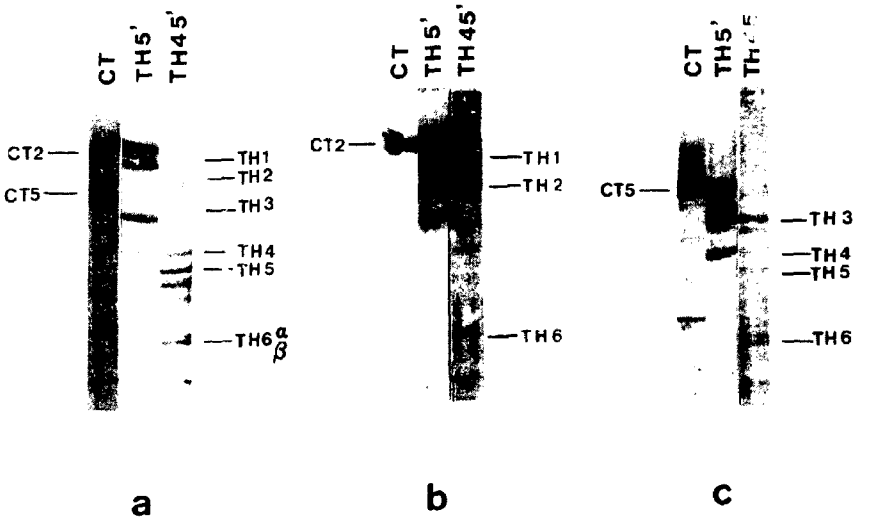

Fig. 7. Immunoblotting of control (a) and specimens with antibodies, anti-CT 2 (b) and anti-CT 5 (c) against chymotryptic digests, CT 2 and CT 5. Control experiment (a) shows the gel pattern before transfer to nitrocellulose membrane. Specimens are shown at the top: the two-headed fragment (CT), and thermolysin digests (digestion time for 5 min (TH 5) and 45 min (TH 45)). Thermolysin or chymotryptic polypeptides are shown at the side.

reacted with antigens of CT 5, TH 3, 4, 5, and 6. The binding reaction indicates that TH 1 and 2 are derived from CT 2, whereas TH 3 and 4 originate from CT 5. The weak reaction of TH 5 and 6 with both antibodies against CT 2 and 5, suggests that the origins of TH 5 and 6 are in both CT 2 and 5. The presence of α and β bands in both TH 5 and 6 is consistent with this suggestion. Each thermolysin digest, TH 5 and 6, separated into α and β bands in the Laemmli system, as previously shown in Fig. 2a. However, the origins of each α and β of TH 5 and 6 remain to be demonstrated clearly, using immunoblotting of the column-purified α and β of TH 5 and 6.

Peptide mapping. We further examined the origin of thermolysin-digested polypeptides by peptides mapping. Specimens were treated with DACM prior to

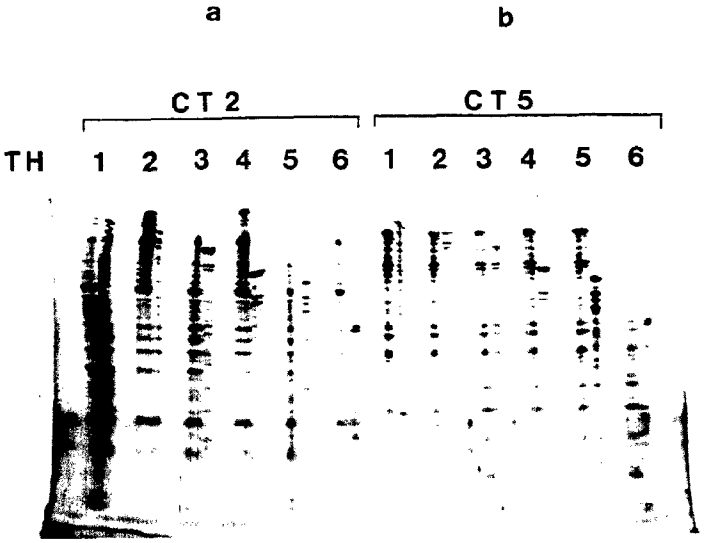

Fig. 8. Peptide mapping of CT 2, CT 5 and thermolysin digested polypeptides, to determine the relationships between CT 2, CT 5 and the digested polypeptides. Each pair consists of the gel of CT 2 or CT 5 at the left, and that of thermolysin digest at the right. Numbers at the upper lane indicate thermolysin polypeptides (TH 1–6).

electrophoresis on SDS-urea and SDS gels, as described previously. Labeled CT 2 and 5, and thermolysin digests were applied to SDS-urea and SDS gels. Bands in the gel were cut out under ultraviolet light illumination. The gel slices were put into the sample slots of stacking gels using the Laemmli system, after each slot was loaded with sample buffer solution containing 5 μg/ml thermolysin. To analyze the peptide maps precisely, two gel slices containing the specimens to be compare were put into the same slot. Fig. 8 shows the peptide mapping patterns of the two specimens to be compared. The patterns of TH 1 and 2 corresponded well with that of CT 2, while the patterns of TH 3 and 4 were almost identical with that of CT 5. The results were consistent with those obtained by immunoblotting, i.e., TH 1 and 2 originate in CT 2, and TH 3 and 4 are derived from CT 5. The patterns of TH 5 and 6 were partially similar to those of CT 2 or 5, which indicates that some parts of TH 5 and 6 are derived from CT 2 or 5. Patterns of SDS and SDS-urea gels of eluted fractions by high pressure liquid chromatography (Fig. 4), showed that some parts of TH 5 and 6, that is TH 5(α) and TH 6(β), have origins in CT 2, and some parts of TH 5 and 6, TH 5(β) and TH 6(α), in CT 5.

Upon consideration of these findings together with the profile of ATPase activity (Fig. 5), we concluded that ATPase activity in fractions 30 to 33 containing TH 2, 5(α) and 6(β) is attributed to CT 2. ATPase activity in fractions 22 to 28, containing TH 4, 5(β), a faint TH 6(α), intermediate and lower bands, results from CT 5. Toyoshima [2] already reported that CT 2 was derived from Aβ HC, while CT 5 originated from Aγ HC. We concluded that the ATPase sites are located in each head of the two-headed fragment which contains Aβ and Aγ HCs.

Discussion

Vale and Toyoshima [13] have recently examined the function of the multiple ATPase heads of 22S dynein by using single- and two-headed fragments of three-headed 22S dynein and observing their motile activity in vitro. The single-headed dynein did not induce movement, even though it was capable of binding to microtubules and hydrolysing ATP. On the other hand, the two-headed dynein translocated microtubules at velocities similar to those measured for intact 22S dynein.

We attempted here to determine the location of the ATPase site in the two-headed fragment by digesting the fragment with thermolysin. The increase in ATPase activity by digestion with proteinases such as trypsin or chymotrypsin has already been reported by Gibbons and Fronk [23] in the flagellar dynein of sea urchin sperm. We also confirmed a gradual increase in ATPase activity during the digestion process of the two-headed fragment with thermolysin. ATPase activity increased to about 6-times the amount present at the beginning of digestion. The increase in ATPase activity of the digested fragment seems to support the idea of 'latency of dynein ATPase activity', proposed by Gibbons and Fronk [23].

Thermolysin at a concentration of 100 μg/ml cut the fragment into several polypeptides; we noted eight polypeptides (TH 1–5α, β and 6α, β) with higher molecular weights. The immunoblotting and peptide mapping in this study showed that TH 1, 2, 5β, and 6α originate in Aβ HC, while TH 3, 4, 5α and 6β are derived from Aγ HC. Based on fractionation by high pressure liquid chromatography and the ATPase activity of the specimens, we concluded that ATPase sites are located in both Aβ HC and Aγ HC in the two-headed fragment. Taking into consideration the results presented by Toyoshima [1], we concluded that *Tetrahymena* 22S dynein had ATPase sites in each heavy chain and each head, that is, Aα HC of the single-headed dynein fragment, and Aβ HC and Aγ HC of the two-headed fragment. Although the translocating ability of the two-headed fragment has already been reported, an interesting problem remains, i.e., to analyze how the two ATPases in the heads collaborate in the translocation of microtubules in vitro.

After the thermolysin-digested fragments were incubated with doublet microtubules, binding ability of the fragments to doublet microtubules was briefly surveyed in the presence or absence of ATP. The gel pattern indicates that a part of TH 4 and 5 bound to the doublet microtubules in ATP-independent manner, which suggests that a part of TH 4 and 5, i.e., a part of Aγ, locate at the ATP-independent binding site, probably at the base of the 22S dynein molecules. However, as Toyoshima [2] previously reported that Aα HC has ATP-independent binding ability, further precise study is still needed.

In our previous work on the sliding velocity of axonemes of sea urchin sperm flagella, a non-linear relationship in a double reciprocal plot was noted, showing a variation of axoneme ATPase activity with Mg-ATP concentration [6]. We suggested that the axonemes might have two ATPases having different K_m values, which would fit the observed non-linear curve of ATPase activity. Tang et al. [15] and Yano-Toyoshima [20], actually showed that the outer dynein arms of sea urchin sperm flagella contain two distinct dynein ATPases, based on the different enzymatic properties of the ATPases in the Aα and Aβ/1C fractions. However, according to Sale and Fox [16], microtubule gliding ability was found in the β-HC-intermediate chain 1 fraction, but neither in the α-HC nor in the intermediate chain 2 and 3 fraction. The single-headed fragment of *Tetrahymena* ciliary dynein

was also reported to have no gliding ability, in spite of ATPase activity [13]. Further study would be of great interest in order to determine the in vivo function of these ATPases which have no gliding ability.

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