Fractionation of Tetrahymena Ciliary Membranes with Triton X-114 and the Identification of a Ciliary Membrane ATPase

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Abstract. Cilia were isolated from Tetrahymena thermophila, extracted with Triton X-114, and the detergent-soluble membrane + matrix proteins separated into Triton X-114 aqueous and detergent phases. The aqueous phase polypeptides include a high molecular mass polypeptide previously identified as a membrane dynein, detergent-soluble α and β tubulins, and numerous polypeptides distinct from those found in axonemes. Integral membrane proteins partition into the detergent phase and include two major polypeptides of 58 and 50 kD, a 49-kD polypeptide, and 5 polypeptides in relatively minor amounts. The major detergent phase polypeptides are PAS-positive and are phosphorylated in vivo. A membrane-associated ATPase, distinct from the dynein-like protein, partitions into the Triton X-114 detergent phase and contains nearly 20% of the total ciliary ATPase activity. The ATPase requires Mg++ or Ca++ and is not inhibited by ouabain or vanadate. This procedure provides a gentle and rapid technique to separate integral membrane proteins from those that may be peripherally associated with the matrix or membrane.

Ciliary and flagellar membranes are associated with a number of metabolic and structural processes, including olfaction (Chen et al., 1986), Ca++ ion regulation (Travis and Nelson, 1986), regulation of ciliary motility (Gustin and Nelson, 1987; Dentler et al., 1980), microtubule assembly (Dentler, 1980a), and gliding (Bloodgood, 1977). Each of these properties require membrane proteins to be properly oriented and localized along the ciliary and flagellar shaft. Little is known about the structure or organization of ciliary membrane proteins but Stephens et al. (1987) proposed that a detergent-resistant membrane skeleton, composed partly of "membrane tubulin," is associated with the cytoplasmic face of scallop gill ciliary membranes and that this skeleton may provide structural rigidity to the membrane as well as attachment sites for the microtubule-membrane bridge proteins (Dentler et al., 1980).

Ciliary membranes and detergent-soluble "membrane + matrix" fractions are composed of 25-50 (or more) different polypeptides, as resolved on one-dimensional SDS-PAGE (Chen et al., 1986; Dentler, 1980b; Adoutte et al., 1980). To understand the functions of these proteins, it is necessary to identify the locations of these proteins in the membrane and identify proteins that may be associated with the membrane skeleton, if such a skeleton exists in all cilia. In this study, Tetrahymena ciliary membranes were fractionated with Triton X-114 to separate and identify integral membrane proteins (Bordier, 1981; Dentler, 1980b; Stephens, 1985a; Pryde and Phillips, 1986) and to determine if the major detergent-soluble proteins, previously identified as tubulins (Dentler, 1980b), are integral membrane proteins. The results show that the major Tetrahymena thermophila membrane proteins migrate at 50 and 58 kD, partition into the Triton X-114 detergent phase and are glycosylated and phosphorylated in vivo. The detergent-soluble Tetrahymena ciliary tubulin partitions into the aqueous phase and is unlikely to be an integral membrane protein.

Since ciliary and flagellar membrane ATPases have been described in a number of organisms (see Dentler, 1981; Travis and Nelson, 1986), the Triton X-114 aqueous and detergent phase fractions were examined for ATPase activity. The results show nearly 20% of the total ciliary ATPase activity is released by Triton X-114, most of which partitions into the detergent phase. The membrane ATPase is insensitive to vanadate and ouabain and requires divalent cations. The function of the ATPase is unknown but it could be a membrane pump or be related to the movements of ciliary surface proteins (Williams et al., 1985; Bloodgood, 1987).

Materials and Methods

Isolation of Cilia and Triton X-114 Fractions

Cilia were isolated from Tetrahymena thermophila, strain B-255 (from E. Oria, University of California, Santa Barbara) using dibucaine as previously described (Suprenant and Dentler, 1988). Cells were grown in 2% proteose peptone (Difco Laboratories Inc., Detroit, MI), 0.1 mM FeCl3, and 0.0025% penicillin-streptomycin in 2.8-liter fernbach flasks at room temperature on an orbital shaking table. For most experiments, 3 liters of cells grown to late log phase, harvested and deciliated in 300 ml of proteose peptone with 300 mg of dibucaine. In some experiments, cells were washed three times in HNMR (50 mM Hepes, pH 6.9, 36 mM NaCl, 1 mM KCl,
0.1 mM MgSO_4, and deciliated in HNMK and 1 mg/ml dibucaine with results identical to those obtained when cells were deciliated in protease. Cells were routinely examined by phase microscopy after deciliation to insure that the cells were intact.

Cilia were washed twice in PEMKS (50 mM Pipes, pH 7.1 with KOH, 3 mM MgSO_4, 0.1 mM EDTA, 50 mM sodium acetate, 1 mM EDTA, 250 mM sucrose) or HEEMS (50 mM Heps, pH 7.1, 3 mM magnesium acetate, 1 mM EGTA, 1 mM EDTA, 250 mM sucrose) as described in the text. DTT (0.1 mM) and 0.4 mM leupeptin were added to the final washed cilia and was included in all subsequent steps. PMSF (29 mM) was prepared in propanol and was added in a 1:1,000 dilution to each solution immediately before use. Washed cilia were suspended in 5–10 ml of PEMKS or HEEMS and were maintained at ice-cold Triton X-14. The suspension was incubated on ice for 20 min and was mixed every 2 min with a Pasteur pipette. Axonemes were then pelleted by centrifugation at 27,000 g for 20 min (4°C) in a rotor (model JA-20; Beckman Instruments Inc., Palo Alto, CA). The supernatant was carefully removed, placed in a conical glass centrifuge tube, and then warmed to 30°C in a water bath for 5 min to induce cloud formation. The sample was centrifuged for 10 min at 1,800 ×_g at room temperature (model Centra 7; International Equipment Co., Needham Heights, MA). The clear aqueous phase was removed from the cloudy detergent phase and each fraction was placed on ice. For most studies, the aqueous phase was extracted two or three times by cooling, adding Triton X-114 to 1% (final concentration), incubating 2–3 min on ice, warming to 30°C, and centrifuging as described above. Greater than three extractions of the aqueous phase did not result in any significant quantity of protein partitioning into the Triton X-114 detergent phase.

For SDS-PAGE or ATPase assays, the Triton X-114 detergent phase sample was diluted with PEMKS to a volume equal to that of the aqueous phase and the diluted sample was placed on ice until all detergent micelles disappeared. Removal of detergent with Bio Beads SM-2 (Bio-Rad Laboratories, Cambridge, MA) was attempted but passage of 5 ml of the diluted detergent phase through three separate 3-ml columns of Bio Beads failed to remove all of the detergent, as judged from the formation of detergent micelles in the eluate at room temperature. Detergent-resistant membrane vesicles were purified by suspending the axoneme + membrane pellet after one extraction of cilia with Triton X-114 in cold PEMKS. The suspension was layered over a sucrose step gradient in HEEMS and were centrifuged for 12 h at 32,000 rpm in a rotor (model SW-41; Beckman Instruments Inc.) at 4°C. Dynein, aqueous phase, and detergent phase samples were run in parallel. Fractions (18 drops [×0.5 ml]) were collected from the bottom of each centrifuge tube. The volume of each fraction varied somewhat depending on the concentration of the detergent. For initial experiments, the protein in each fraction was detected by reading the absorbance at 280 nm but this was discontinued because the Triton X-114 generally condensed at room temperature and interfered with the absorbance measurements.

Assays were run at room temperature. 2-ml samples were layered over a 5–20% (wt/wt) linear sucrose gradient in HEEM and were centrifuged for 12 h at 32,000 rpm in a rotor (model SW-41; Beckman Instruments Inc.) at 4°C. Dynein, aqueous phase, and detergent phase samples were run in parallel. Fractions (18 drops [×0.5 ml]) were collected from the bottom of each centrifuge tube. The volume of each fraction varied somewhat depending on the concentration of the detergent. For initial experiments, the protein in each fraction was detected by reading the absorbance at 280 nm but this was discontinued because the Triton X-114 generally condensed at room temperature and interfered with the absorbance measurements.

### In vivo ³²P-labeling and Autoradiography

Cells were grown to ~10⁵ cells/ml in 2% proteose peptone, harvested by centrifugation, and washed into 1.3 liter of sterile HNMK supplemented with 1 mM glucose, 0.0025% penicillin-streptomycin. Carrier-free ³²P (as phosphate) was added (1 nCi) and cells were incubated on a shaker at room temperature for 17 h. Cells were harvested by centrifugation, suspended in 2% proteose peptone, and cilia were amputated and isolated as described above. Cilia were demembranated with NP-40 or with Triton X-114, and the various membrane and matrix fractions were separated as described above. Samples were loaded and run on 15–20% thick 4–16% polyacrylamide slab gels (see below). Gels were fixed following the method of Fairbanks et al. (1971). The inclusion or omission of Coomassie Blue from the gels had no effect on the labeling patterns observed, although both stained and unstained gels were autoradiographed. After fixation, gels were dried onto filter paper using a gel drier (model 443; Bio-Rad Laboratories). Dried gels were autoradiographed with pre-flashed X-O Mat film (Eastman Kodak Co., Rochester, NY) and a Cronex Lightning Plus intensifying screen (DuPont Co., Wilmington, DE). Typically, films were exposed at ~80°C for 7 d and developed for 6 min in D-19.

### Sucrose Gradient Separation of Dynein and Detergent-released Polypeptides

Triton X-114 aqueous and detergent phase ciliary fractions were prepared in HEEMS as described above. Axonemal dynein was prepared by suspending the axonemes in HEEM and then adding NaCl to 0.6 M. The suspensions were incubated on ice for 30 min and the axonemes were pelleted for 25 min at 40,000 g, 4°C. The best resolution of ATPase peaks was obtained if the NaCl supernatant was dialyzed against 200 vol of cold HEEM for 3 h followed by centrifugation at 48,000 g for 15 min at 4°C to remove precipitated material. 2-ml samples were layered over a 5–20% (wt/wt) linear sucrose gradient in HEEM and were centrifuged for 12 h at 32,000 rpm in a rotor (model SW-41; Beckman Instruments Inc.) at 4°C. Dynein, aqueous phase, and detergent phase samples were run in parallel. Fractions (18 drops [×0.5 ml]) were collected from the bottom of each centrifuge tube. The volume of each fraction varied somewhat depending on the concentration of the detergent. For initial experiments, the protein in each fraction was detected by reading the absorbance at 280 nm but this was discontinued because the Triton X-114 generally condensed at room temperature and interfered with the absorbance measurements.

### ATPase and Protein Assays

ATPase activities were carried out using the method of Atkinson et al. (1973). The assay is sensitive and reproducible but is sensitive to labrol impurities. Consistent results were obtained with Surfact-Amps PX (a 10% labrol solution obtained from Pierce Chemical Co., Rockford, IL). Assays were run at room temperature in 50 mM Heps, pH 7.1, 3 mM magnesium acetate, 0.5 mM EGTA, 0.1 mM DTT, and 0.4 mM leupeptin. Assays were started by adding 2 mM ATP and were carried out at room temperature for 30 min. For SDS-PAGE, 100 μl aliquots of each sample were loaded on a 20% well separation gel.

### Electrophoresis

SDS-PAGE was carried out with a modification of the discontinuous system of Laemmli (1970). Separation gels were composed of a continuous gradient of 4–16% acrylamide and were adjusted to pH 8.8 or 8.3 with HCl immediately before pouring the gels. Gels were run at constant current in a slab gel apparatus (model SE-600; Hoefer Scientific Instruments, San Francisco, CA) at 10°C and were fixed and stained using Coomassie Blue (Fairbanks et al., 1971) or Silver (Pratt et al., 1984). Gels were photographed with Technical Pan film (Eastman Kodak Co.) developed in HC-110. Molecular mass standards included myosin (205 KD), β-galactosidase (116 KD), phosphorylase B (97 KD), BSA (66 KD), egg albumin (49 KD), and carbonic anhydrase (29 KD). PAS staining was carried out by a modification of the method of Pierce Chemical Co.).

### Protein concentrations were determined using the method of Bradford (1976) or the BCA assay by Pierce (Pierce Chemical Co.).

### Electrophoresis

Proteins were blotted from unfixed gels onto nitrocellulose using the procedure of King et al. (1986). Nitrocellulose sheets were cut into strips, incubated with tubulin antibodies for 1 h at room temperature, and the tubulin antibodies were visualized with peroxidase goat anti-mouse IgG (model No. 62-6520; Zymed Laboratories, San Francisco, CA). Monoclonal tubulin antibodies that bind to Tetrahymena β tubulin were obtained from ICN ImmunoBiologicals (Lisle, IL; Clone No. DM-1A) and to Tetrahymena β tubulin was identified with Tu9B obtained from Dr. L. I. Binder, University of Alabama.

Gels were scanned (model No. CS-930 scanner; Shimadzu Scientific In-
Polypeptide Composition of the Membrane + Matrix

Extraction of Tetrahymena cilia with PEMKS and NP-40 reveals the major 55-kD polypeptides and high molecular mass dynein-like polypeptides as previously reported (Fig. 1, NA and NS) (Dentler, 1980b; Dentler et al., 1980). To further fractionate the detergent-soluble proteins and identify those likely to be integral membrane proteins, cilia were extracted with PEMKS and Triton X-114 at 4°C, axonemes were pelleted, and the supernatant warmed to 30°C to separate the hydrophobic detergent phase from the soluble aqueous phase. Analysis of the fractions with SDS-PAGE (Fig. 1, TA, TS, and TP) reveals that the major polypeptides released into NP-40 are resolved into at least four major polypeptides, two of which partition into the detergent phase while two to four polypeptides remain in the aqueous phase. Repeated extractions of the aqueous phase with Triton X-114 result in the sedimentation of progressively lower quantities of protein but, regardless of the number of extractions, each detergent phase sample contains the same number and ratio of polypeptides, as resolved by SDS-PAGE (Fig. 1, TP).

Identification of the major membrane polypeptides by SDS-PAGE depends on the pH of the separation gel and the staining method employed. As judged by Coomassie Blue staining, the major Triton X-114 detergent phase polypeptides migrate with apparent molecular masses of 50 and 58 kD in pH 8.6 separation gels. Only the 58-kD polypeptide stains with silver (Figs. 1 and 2). The remainder of the polypeptides that partition into the Triton X-114 detergent and aqueous phases stain equally well with Coomassie Blue and silver, with the exception of the high molecular mass membrane vesicle protein whose staining is slightly enhanced by silver staining (Fig. 2).

Densitometry of Coomassie Blue-stained gels reveal that the major 50- and 58-kD polypeptides comprise 25 and 65% of the Triton X-114 detergent phase protein, respectively (Table I). The remaining polypeptides, which comprise <10% of the detergent phase protein, include those of 126, 104, 69, 49, and 29 kD (see Fig. 7). The 50- and 58-kD polypeptides comprise ~3 and 7%, respectively, of the total ciliary protein. Nearly half of the total Triton X-114 solubilized protein remains in the aqueous phase. The major aqueous phase polypeptides migrate in a broad band at 50–60 kD (Figs. 1 and 2) and include bands that comigrate with α and β tubulins and bands that comigrate with the detergent phase polypeptides. Small quantities of the detergent phase 50- and 58-kD polypeptides remained in the aqueous phase after repeated extractions with Triton X-114 and the 69- and 29-kD polypeptides are found in both detergent and aqueous phases. A small quantity of detergent-resistant membrane vesicles are present in the axoneme fractions (data not shown). These vesicles were purified from the axoneme fractions on sucrose gradients and contain the major detergent phase polypeptides and the same minor bands present in the Triton X-114 detergent phase plus a high molecular mass polypeptide that migrated more slowly than the dynein heavy chains from axonemal or aqueous phase fractions (Fig. 2).

Tubulins were identified by immunoblots of detergent and aqueous phase polypeptides with α and β tubulin antibodies. To compare these results with those described in an earlier study, cilia were extracted with NP-40, which releases α and β tubulins (Figs. 3 and 4). Tubulins are present in the NP-40 fraction.

Table I. Composition of Tetrahymena “Membrane + Matrix”

<table>
<thead>
<tr>
<th>Triton X-114</th>
<th>Percent of fraction</th>
<th>Percent of total ciliary protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axonemal tubulin</td>
<td>41</td>
<td>32</td>
</tr>
<tr>
<td>Axonemal dynein (HMW)</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Total protein</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Triton X-114 aqueous phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acceous 55 kD + tubulin</td>
<td>28</td>
<td>3</td>
</tr>
<tr>
<td>Total protein</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Triton X-114 detergent phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>55 kD</td>
<td>65</td>
<td>7</td>
</tr>
<tr>
<td>50 kD</td>
<td>25</td>
<td>3</td>
</tr>
</tbody>
</table>

The polypeptide composition of the major ciliary axonemal and membrane proteins as determined from scans of Coomassie Blue-stained gels. Based on molar ratios, the 55- + 58-kD polypeptides are present in 2–6× greater quantities than ciliary dynein.
Figure 2. Comparison of the major Triton X-114 axonemal (A), aqueous phase (S), and detergent phase (P) polypeptides with those of purified membrane vesicles (MV). Cilia were extracted in PEMKS and separated at pH 8.3. Molecular mass markers are shown in the left lane and identical gels are stained with Coomassie Blue (Coo B) and silver (Silver). The membrane vesicles are enriched in a high molecular mass polypeptide that migrates slightly higher than does any of the axonemal dyneins or the membrane dynein that partitions into the Triton X-114 aqueous phase (compare S with MV). Other than the high molecular mass polypeptide, the composition of the membrane vesicles is identical to that of the Triton X-114 detergent phase.

Identification of Glycosylated and Phosphorylated Membrane Polypeptides

Previous studies reported that ciliary membrane tubulin is glycosylated (Stephens, 1977; Dentler, 1980b), so samples of the Triton X-114 detergent and aqueous phase polypeptides were separated by SDS-PAGE and were stained using the PAS procedure. The results (Fig. 4) show that the major PAS-positive bands in the NP-40 extracts partition into the Triton X-114 detergent phase. At the highest sensitivity of the densitometer, the axonemes contained only a trace of PAS-positive material. Trace amounts of PAS-positive material were present in the Triton X-114 aqueous phase but it is unclear whether this is due to glycosylated tubulin or to incompletely separated detergent phase polypeptides. These results show that the detergent-phase polypeptides are glycosylated and the major 50- and 58-kD glycosylated ciliary membrane proteins are not tubulins.

Since tubulin is associated with the detergent-soluble membrane fraction and since phosphorylated tubulin can bind to lipid vesicles in vitro (Hargreaves et al., 1986), Tetrahymena cells were grown with $^{32}$P and isolated cilia examined to determine if the detergent-solubilized tubulin is phosphorylated in vivo. The NP-40 soluble fractions contained two phosphorylated polypeptides of 50 and 58 kD (Fig. 5) as well as material at the leading edge of the gel, presumably phospholipids. Fractionation of the cilia with Triton X-114 reveals that most of the phosphorylated protein partitions...
Figure 4. Scans of Coomassie Blue-stained gels (Coo B), identification of PAS-positive bands (PAS), and immunoblots stained with α (α) and β (β) tubulins. Cilia were extracted in PEMKS and polypeptides separated on pH 8.25 gels. Triton X-114 detergent phase (DP), aqueous phase (AQ), and axonemes (AX) are compared with NP-40 (NP40) supernatants (S) and axonemes (AX). The tops of each gel is toward the left. The major PAS-positive ciliary proteins migrate in the 50–58-kD region of detergent-soluble proteins but not in the axonemes. Most of the PAS-positive polypeptides found in the detergent extracts partition into the Triton X-114 detergent phase. In contrast, all of the α and β tubulin in the detergent extracts partition into the Triton X-114 aqueous phase. Tubulins are not, therefore, the major PAS-positive ciliary proteins.

Selective Extraction of an ATPase Activity in the Triton X-114 Detergent Phase

NP-40 extracts of Tetrahymena cilia separated on sucrose gradients were previously shown to contain two ATPase activities, one of which is a dynein-like protein that sediments at 14 S and contains polypeptides that comigrate with dynein and another ATPase is composed of polypeptides that migrate at ~55 kD (Dentler et al., 1980). Since most of the high molecular mass detergent-soluble polypeptides partition into the Triton X-114 aqueous phase and the major ~55-kD detergent-soluble polypeptides partition into the detergent phase, the aqueous and detergent phase fractions were assayed to determine if the detergent phase polypeptides are ATPases.

Both the detergent and aqueous phase fractions contain ATPase activity (Table II) and the total detergent-soluble protein contains nearly 20% of the total ciliary activity. To identify the polypeptides responsible for the ATPase activity, Triton X-114 aqueous and detergent phase ciliary fractions were separated on sucrose gradients and were compared with salt-extracted axonemal dynein. In some experiments, cilia were washed and extracted with Triton X-114 in PEMKS and proteins were separated on linear 10–40% sucrose gradients (not shown). The Triton X-114 aqueous phase contains the high molecular mass ATPase that cosediments with 14-S dynein as well as a second activity that remains near the top of the gradient. All of the ATPase activity in the Triton X-114 detergent phase remains near the top of the gradient and contains polypeptides identical to those found at the top of the aqueous phase gradients.

Figure 5. In vivo phosphorylation of ciliary polypeptides separated by NP-40 and Triton X-114. Cells were labeled with inorganic 32P, cilia were isolated, extracted with 1% NP-40 in PEMKS, and polypeptides were separated by SDS-PAGE at pH 8.8 (left pair of gels). NS, NP-40 soluble protein; NA, axonemes. Triton X-114 solubilized proteins were warmed, separated into axoneme (TA), aqueous phase (TS), and detergent phase (TP) polypeptides on pH 8.4 gels (right pair of gels) which do not separate the 50- and 58-kD polypeptides. Coomassie Blue-stained gels (Coo B) and autoradiograms (32P) are shown. The major phosphorylated polypeptides are found in the detergent-soluble extracts and comigrate with axonemal tubulins but partition into the Triton X-114 detergent phase, which does not contain tubulin. The labeled polypeptides that comigrate with tubulin in the axoneme fraction may be due to detergent-insoluble membrane vesicles (see text). Molecular mass markers on the pH 8.4 gels are (top to bottom) 116, 97, 66, 49, and 29 kD.
When isolated cilia are washed and demembranated in HEEMS, which is lower ionic strength than PEEMKS, little dynein is released. Separation of the ATPase activity on sucrose gradients reveals a single ATPase peak with virtually identical sedimentation rates in the Triton X-114 aqueous and detergent phases (Fig. 6, D and F). The dialyzed ciliary dynein is separated into 14- and 22-S peaks (Fig. 6 B). The polypeptide composition of the major ATPase peaks, from a 10–40% sucrose–HEEM gradient, is shown in Fig. 7. Neither of the dynein peaks contain bands that comigrate with the proteins released by Triton X-114 in HEEMS. Both the aqueous and detergent phases contain the major 50- and 58-kD polypeptides as well as minor polypeptides of 69 and 104 kD.

The polypeptide composition of the detergent and aqueous phase ATPases isolated in HEEMS are similar, but not identical. To determine if the same ATPase is present in each fraction, the effects of inhibitors, divalent cations, and nucleotides were examined. Neither the aqueous nor the detergent phase ATPase activities are inhibited by 10 μM vanadate and up to 100 μM vanadate only slightly inhibits the activity. By contrast, sucrose gradient–purified 14- and 22-S dynein ATPase was almost completely inhibited by 10 μM vanadate. The effect of ouabain was examined to identify possible ciliary membrane Na+/K+ ATPases and the results of two experiments showed that up to 5 mM ouabain had no effect on the ATPase activity of the aqueous or detergent phase enzyme. The nucleotide specificities of the Triton X-114 aqueous and detergent phase ATPase activities are essentially identical (Table III). Each exhibit a preference for ATP but require divalent cations. The Ca2+ and Mg2+ activities are nearly identical at 1 mM (Table III) but higher concentrations of Ca2+ show a slight, but reproducible, decline in the ATPase activity (Fig. 8).

The Km's for the Triton X-114 aqueous and detergent phase ATPases are 100 and 93 μM, respectively (Fig. 9). Cilia were also isolated and extracted with HEEMS–NP-40 and the Km of the detergent-released ATPase was 90 μM (Fig. 9). Therefore, the same ATPase activity is present in cilia extracted with HEEMS and either NP-40 or Triton X-114. These data indicate that the same ATPases are present in both the aqueous and detergent phase fractions isolated from HEEMS–Triton X-114 extracted cilia. They have virtually identical requirements for Ca2+ and Mg2+, similar Km's, both are insensitive to vanadate and ouabain, and the peaks of ATPase activity eluted from sucrose gradients contain the same major polypeptides.

**Table II. Ciliary Axoneme and Membrane Fraction ATPase Activities**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific activity</th>
<th>Total activity</th>
<th>Percent of total ciliary ATPase</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>nM P. min⁻¹ mg⁻¹</td>
<td>Sp Act x mg protein</td>
<td></td>
</tr>
<tr>
<td>Axonemes</td>
<td>1,800</td>
<td>46,800</td>
<td>81</td>
</tr>
<tr>
<td>Triton X-114</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aqueous phase</td>
<td>519</td>
<td>908</td>
<td>2</td>
</tr>
<tr>
<td>detergent phase</td>
<td>6,940</td>
<td>10,173</td>
<td>17</td>
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**Discussion**

Triton X-114 is a useful tool with which to solubilize membranes and separate hydrophobic integral membrane proteins from those that are more water soluble (Bordier, 1981). Previous studies of ciliary membrane proteins solubilized with Triton X-114 found relatively few polypeptides partitioning into the detergent phase. The detergent phase fraction from scallop ciliary membranes contains a small amount of a 20-kD protein (Stephens, 1985a) and the detergent phase of frog olfactory cilia contains only one 95-kD protein (Chen et al., 1986). When applied to *Tetrahymena* ciliary membranes, the majority of detergent-soluble polypeptides remain in the aqueous phase while only a few polypeptides, that comprise nearly 50% of the total detergent-soluble protein, are integral membrane proteins. One advantage to this method is that it separates detergent-soluble tubulin from two major integral membrane proteins that comigrate with tubulin in one-dimensional SDS-PAGE (Dentler, 1980b). With the separation of these polypeptides, the major phosphorylated and glycosylated membrane polypeptides can now be identified and at least one previously unidentified ciliary membrane ATPase is now partially purified. The major detergent phase polypeptides are exposed to the cell surface since they are PAS-positive. Furthermore, these polypeptides are labeled in living cells with a biotinylated probe that binds cell surface but not axonemal proteins (Dentler, W. L., and N. Fragnito, manuscript in preparation). The observation that the major membrane polypeptides are phosphorylated may reveal a role of phosphorylation/dephosphorylation in the membrane protein function or may reveal their association with phospholipids. The role of phosphorylation remains to be determined.

The initial impetus for this study was to characterize membrane-associated tubulin in *Tetrahymena* and to determine if phosphorylation may regulate its association with the membrane, as suggested by Hargreaves et al. (1986). Tubulins are found in detergent-soluble extracts of scallop gill cilia and *Tetrahymena* cilia (Stephens, 1977, 1983, 1985b, 1986; Dentler, 1980b) and proteins that comigrate with tubulins are present in *Tetrahymena* ciliary membrane vesicles (Dentler, 1980b). The major 55-kD detergent-soluble polypeptides are phosphorylated in vivo, which is consistent with tubulin phosphorylation being involved in its association with the membrane. However, Triton X-114 fractionation of the detergent-soluble polypeptides revealed that the major 50- and 58-kD polypeptides partition into the detergent phase and these polypeptides, not tubulin, that is phosphorylated in vivo.

Is tubulin associated with *Tetrahymena* ciliary membranes? The results reported here are consistent with those reported by Stephens (1985a), in that the detergent-soluble tubulin partitions into the Triton X-114 aqueous, but not detergent phase. These results indicate that tubulin is unlikely to be an integral membrane protein but they do not prove that it is not associated with the membrane since some chromaffin granule membrane proteins partition into the aqueous phase (Pryde and Philips, 1986). It is unlikely that the tubulin is freely soluble in the intact cilia. Examination of thin sections of isolated cilia fixed before detergent extraction revealed that virtually all ciliary membranes were broken open and, although attached to the axonemes, they were not sufficiently intact to prevent the release of soluble tubulin or...
Figure 6. Analysis of ciliary fractions isolated in HEEMS plus Triton X-114. Fraction No. 1 was at the bottom of each gradient. Salt-extracted and dialyzed dynein is shown in A and B. Triton X-114 aqueous (C and D) and detergent (E and F) phases are shown in E and F. ATPase activity is expressed in nmols P$_\text{i}$ released/min. Each fraction of the dynein (25 µl/assay), aqueous phase (25 µl/assay), and detergent phase (10 µl/assay) was assayed for ATPase activity and 100 µl aliquots of each fraction was assayed by SDS-PAGE. The 22- S (fraction 6) and 14-S (fraction 14) dynein ATPases were clearly resolved (A and B). Since extraction in HEEMS does not release the membrane dynein seen in Fig. 1, the peaks of ATPase activity in both the aqueous and detergent phases sediment near the top of the gradient and contain the same polypeptides. Comparison of the polypeptides found in the peaks of ATPase activity is shown in Fig. 7.

any other soluble ciliary protein (data not shown). Despite the presence of disrupted membranes and an exposed matrix, the tubulins (and other aqueous phase polypeptides) were only released from the axonemes by the addition of detergent. Some of the soluble tubulin could be due to microtubule breakdown, since examination of thin section axonemes after detergent-extraction showed a small amount of microtubule breakdown and a portion of the soluble tubulin was assembled into microtubules in vitro upon addition of taxol (data not shown). However, the amount of released tubulin is greater than that expected from the examination of thin section axonemes after detergent extraction (see Stephens, 1986). These results do not, therefore, rule out the possibility that tubulin is associated with Tetrahymena ciliary membranes but they do show that, contrary to a previous report (Dentler, 1980b), the detergent-soluble tubulins are not the
Table III. Nucleotide and Ion Specificities of Ciliary ATPases

<table>
<thead>
<tr>
<th>Fraction</th>
<th>ATP/GTP</th>
<th>ATP/CTP</th>
<th>ATP/UTP</th>
<th>Mg(^{2+})/Ca(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axonemes</td>
<td>1.7</td>
<td>0.6</td>
<td>1.0</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>Triton X-114</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aqueous phase</td>
<td>2.1</td>
<td>1.5</td>
<td>1.3</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>detergent phase</td>
<td>2.6</td>
<td>1.3</td>
<td>1.0</td>
<td>1.2 ± 0.1</td>
</tr>
</tbody>
</table>

Comparison of the specific Mg\(^{2+}\)-ATPase activities and divalent cation requirements for ciliary ATPases. Three different preparations of each fraction were tested for nucleotide specificity in the presence of 2 mM magnesium acetate. Mg\(^{2+}\)/Ca\(^{2+}\) stimulation of ATPase activity was determined for 12 axoneme preparations and 16 Triton X-114 aqueous and detergent phase fractions.

The presence of a substantial amount of ciliary and flagellar membrane-associated ATPase activity has been described in other biochemical (Gibbons, 1965; Travis and Nelson, 1986) and cytochemical studies (see Dentler, 1981) but neither the identity nor the function of the membrane-associated ATPases are understood. There are at least two detergent-soluble *Tetrahymena* ciliary membrane ATPases, one of which has been identified as a high molecular mass dynein-like protein forming a bridge linking the doublet microtubules to proteins in the ciliary membrane (Dentler et al., 1980) and partitions into the Triton X-114 aqueous phase. The release of the dynein-like protein from the axonemes depends on the buffer composition, since it could be released in PEMKS but little of the high molecular mass ATPase was released in HEEMS. Based on its solubility, the high molecular mass ATPase is probably most tightly associated with the axonemes. Since it does not partition into the detergent phase it is unlikely to be an integral membrane protein.

The second ATPase is composed of lower molecular mass polypeptides and partitions into the Triton X-114 detergent phase.
phase, which strongly suggests that it is an integral mem-
brane protein. Preliminary identification of the polypeptides 
comprising the ATPase can be made by comparing the polypeptide composition of the peaks of ATPase activity in the 
detergent and aqueous phases in HEEMS-extracted cilia, be-
cause the ATPases in each phase had essentially identical 
Km's, Ca++ and Mg++ stimulation, sensitivity to vanadate 
and ouabain, and sedimentation rate in sucrose gradients. In 
10 separate experiments, the only major polypeptides that 
were common to both preparations were the 50- and 58-kD 
bands and a relatively minor 69-kD band. Attempts to sepa-
rate the ATPase activity from Triton X-114 or from the major 
bands and a relatively minor 69-kD band. Attempts to sepa-
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rate the ATPase activity from Triton X-114 or from the major 

The function of the detergent phase ATPase is unknown. 
One obvious role of a membrane-associated ATPase is to 
regulate ion flux across the ciliary membrane. Ca++-stimu-
lated ATPase activities have been reported in membrane or 
detergent extracts of Tetrahymena cilia (Satter, 1976), Para-

Figure 9. Lineweaver-Burke plots comparing the Km of the 
Mg++-ATPase activity released from cilia with HEEMS and 
NP-40 (NP) with that found in the HEEMS-aqueous (AP) 
detergent (DP) phases. Each of the preparations had 
essentially equal Km's, which strongly suggests that identi-
cal enzymes are found in each preparation.

phase, which strongly suggests that it is an integral mem-
brane protein. Preliminary identification of the polypeptides 
comprising the ATPase can be made by comparing the polypeptide composition of the peaks of ATPase activity in the 
detergent and aqueous phases in HEEMS-extracted cilia, be-
cause the ATPases in each phase had essentially identical 
Km's, Ca++ and Mg++ stimulation, sensitivity to vanadate 
and ouabain, and sedimentation rate in sucrose gradients. In 
10 separate experiments, the only major polypeptides that 
were common to both preparations were the 50- and 58-kD 
bands and a relatively minor 69-kD band. Attempts to sepa-
rate the ATPase activity from Triton X-114 or from the major 
ciliary polypeptides contained in the Triton X-114 detergent 
phase were not successful. The major ciliary membrane 
ATPase may, therefore, be part of a complex containing the 
other detergent phase polypeptides (also see Dentler et al., 
1980). Identification of the ATPase will depend on further 

purification or photoaffinity labeling.

Are these ATPases found in all cilia and flagella? The "membrane dynein" has only been identified in detergent ex-
tracts of scallop gill and Tetrahymena cilia (Dentler et al., 
1980) so this report merely confirms its presence and puri-
ification from Tetrahymena. The detergent phase ATPase may 
be present in Tetrahymena but not in scallop gill cilia, since 
a comparison of the NP-40 soluble protein separated on su-
crose gradients revealed a peak of ATPase that coexisted 
with 55-kD polypeptides in NP-40 extracts of Tetrahymena 
but not in scallop gill cilia (Dentler et al., 1980). If the deter-
gen phase ATPase is present in Tetrahymena but not in scal-
lop ciliary membranes, then the function of the ATPase may 
reflect different activities that occur along the ciliary surface 
of these two organisms. Since the extraction procedures de-
scribed in this report are simple and can be carried out on 
small quantities of cilia, confirmation of similar ATPase ac-
tivities in other cilia and flagella should easily be obtained.

The Triton X-114 detergent phase polypeptides are major 
ciliary components: they comprise nearly 7% of the total 
ciliary protein by weight and are present in two to five times 
the quantity of the (axonemal) dynein heavy chains, based 
on molar ratios. It is likely that they serve important func-
tions in the structure or function of ciliary membranes, 
including cell recognition and the movements of material 
within or on the surface of the ciliary membrane. The pres-
ence of an ATPase associated with these major proteins indicates that they may be important for either ion transport or cell-surface motility. If similar proteins are found in other ciliary membranes, the fractionation methods described here should be useful to isolate them for further analysis.

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References


