# Isolation of a Sixth Dynein Subunit Adenosine Triphosphatase of *Chlamydomonas* Axonemes

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***Abstract.*** This study of the axoneme led to the identification of a previously unknown adenosine triphosphatase (ATPase), which is likely a major component of inner dynein arms. The ATPase was isolated from a soluble fraction of axonemes obtained from *pf* 28, a *Chlamydomonas* mutant lacking the outer dynein arms. The activity hydrolyzed up to 2.3 μmol of ATP·min$^{-1}$·mg$^{-1}$ of protein (at pH 7.2, in the presence of both $Ca^{++}$ and $Mg^{++}$), had a sedimentation coefficient of 11S in sucrose gradient, and cosedimented with four polypeptides of apparent molecular weight 325,000, 315,000 140,000, and 42,000. Several arguments indicate that the new ATPase is a component of the inner dynein arms. Three or four polypeptides cosedimenting with the activity belong to a group of axonemal components that are deficient in the axonemes of *pf* 23 and *pf* 30, two mutants that display different levels of inner dynein arm deficiency. The 42,000 component is axonemal actin, a subunit of two other inner dynein ATPases. The two polypeptides of molecular weight >300,000 have electrophoretic mobility similar to that of high molecular weight components of outer and inner dynein arms. In spite of some similarities each ATPase isolated from inner or outer arms is composed of a different set of polypeptides. Different ATPases may be required for the modulation of localized sliding of adjacent outer double microtubules in the axoneme.

THE outer and inner dynein arms generate the movement of cilia and flagella from their specific location on the microtubules of the axoneme. Studies of dyneins of sea urchin sperm, *Tetrahymena cilia*, and *Chlamydomonas* flagella have mainly focused on the morphology of dynein arms in situ and in vitro, on the identification of molecular components and ATPase activities of dynein arm substructures, on the analyses of formation and ATP-dependent dissociation of microtubule–dynein complexes. The literature on dyneins that describes these major subjects of investigation has been recently reviewed (2, 6, 21, 22).

Analyses of axonemal polypeptides of *Chlamydomonas* mutants, partially deficient or lacking outer or inner arms, allowed the identification of 13 outer and 10 inner dynein arm components (5). Many of these polypeptides are ATPase subunits. In fact, proteins extracted from the axonemes of wild-type cells with a high concentration of NaCl comprised five different ATPase activities carried out by dynein arm components (13, 14, 19, 20). Each ATPase is formed by at least one polypeptide of molecular weight >300,000 (HMW),[1] which may contain sites responsible for ATP binding (15) and be phosphorylated (20). Three outer dynein arm ATPases have completely different molecular composition and two inner dynein arm ATPases are assembled with the same polypeptides of low molecular weight, including an axonemal form of actin (18).

Additional ATPase activities located in the inner dynein arms may exist. In fact three HMW subunits of dynein arms have not been identified and analyzed after isolation. Moreover the morphology of inner dynein arms suggests the existence of two kinds of inner arms, each composed of two or three substructures bridging A and B microtubules (3). These substructures, like those of outer dynein arms, may be a site for ATPase activity occurring during the dissociation of microtubule–dynein linkages (6, 12).

Further studies of inner dynein arms could be developed with two *Chlamydomonas* mutants, *oda*38 and *pf* 28 (4, 7, 10), each missing outer dynein arms but apparently normal in the remaining axonemal structures. The purification of a previously unknown ATPase activity from the axonemes of the mutant *pf* 28 is described here. Four inner dynein arm components were copurified with the activity and two of them are of molecular weight >300,000.

## Materials and Methods

*Chlamydomonas reinhardtii* mutant *pf* 28 was characterized by Mitchell and Rosenbaum (10). The mutant *pf* 30 was isolated and characterized by Bessie Huang, Zenta Ramanis, and David Luck (Rockefeller University, NY) and further analyzed by Brokaw and Kamiya (1). The axonemes of this mutant are partially deficient of inner dynein arms (1) and are missing two HMW components, III and IV. Mating type + gametes of strain 137c, *pf* 28, and *pf* 30 were cultured and labeled with [$^{35}$S]sulphuric acid as described by Luck et al. (9) except that the radioactivity used was 25 mCi/liter of medium. Axonemes were prepared following the procedure of Huang et al. (5). Extraction of polypeptide components from the axonemes in the pres-

1. *Abbreviations used in this paper*: HMW, molecular weight >300,000.

*Table I. Solutions Used for Gel Electrophoresis of HMW Dynein Components*

| Solution | Stacking gel | Resolving gel 3.2% | Resolving gel 5% | Cathodic buffer | Anodic buffer |
|---|---|---|---|---|---|
| 1 | 1.87 ml | 4.79 ml | 7.52 ml | | |
| 2 | | 9 ml | 9 ml | | 200 ml |
| 3 | 5 ml | | | | |
| 4 | | | | 50 ml | |
| 5 | | | | 5 ml | |
| 6 | | 3.8 ml | | | |
| 7 | 0.2 ml | 0.45 ml | 0.45 ml | | |
| $H_2O$ | 12.5 ml | 26.71 ml | 6 ml | 944 ml | 800 ml |
| β-Met* | | | | 1 ml | |
| TEMED‡ | 20 μl | 20 μl | 10 μl | | |
| AP§ | 0.32 ml | 0.14 ml | 20 μl | | |
| Urea | | | 21.62 g | | |
| Total volume | 20 ml | 45 ml | 45 ml | 1 liter | 1 liter |

Solutions 1–7 are described in Materials and Methods.
* β-Mercaptoethanol.
‡ *N,N,N',N'*-tetramethyl-ethylenediamine.
§ 12.5% ammonium peroxydisulfate solution was prepared daily.

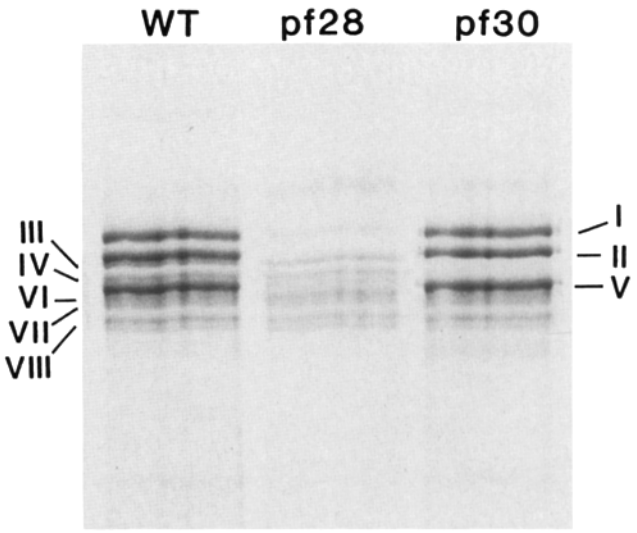


*Figure 1.* Electrophoretograms of axonemal polypeptides of wild-type, mutant *pf* 28 and mutant *pf* 30 cells were obtained by autoradiography of $^{35}$S-labeled polypeptides of molecular weight >300,000. Dynein arm components III, IV, VI, VII, and VIII are indicated in the electrophoretogram of polypeptides of wild-type axonemes. Outer dynein arm components I, II, and V are indicated in the electrophoretogram of polypeptides of *pf* 30 axoneme.

ence of NaCl was performed as in Piperno and Luck (19). Protein, ATPase, GTPase assays, chromatography on hydroxyapatite in the presence of 0.5 M NaCl, and two-dimensional gel electrophoresis were as described in Piperno and Luck (20). ATPase or GTPase assays were also performed in 0.15 M Tris.Cl, pH 7.2, 4 mM $MgCl_2$, plus 1 mM EGTA or 0.5 mM $CaCl_2$. The unit of ATPase activity was defined as the amount of enzyme which hydrolyzes 1 μmol of Pi per minute. Protein content of chromatographic or sucrose gradient fractions was calculated from their $^{35}$S radioactivity and the specific radioactivity of the high salt axonemal extract by making the assumption that specific radioactivity was equal for all polypeptides. Immunoblots were performed as in Piperno and Fuller (17). Transfer to nitrocellulose of polypeptides of molecular weight >300,000 was performed at 400 mA for 7 h. The monoclonal antibodies 18βC and 18βB (8), specific for *Chlamydomonas* outer dynein arm component II, were kindly provided by Dr. Stephen King of the Worcester Foundation for Experimental Biology, Shrewsbury, MA. Monoclonal antibodies 582-1 and 569-4, specific for outer dynein arm component V, are produced by hybridomas derived from the fusion of the myeloma $P_3U_1$ with spleen cells from BALB/c mice immunized with all HMW components of wild-type axonemes. HMW components of wild-type axonemes were prepared by gel electrophoresis, eluted from the gel, and injected into the peritoneum with incomplete Freund adjuvant. Selection and growth of hybridomas were as previously described (16). The specificity of 582-1 and 569-4 was determined with immunoblots of axonemal polypeptides from wild-type cells and dynein arm-deficient mutants (5).

### *Electrophoresis in Polyacrylamide Gels of Dynein HMW Components*

Electrophoresis and separation of dynein HMW components was performed as in previous studies (19) following a modified version of the method of Neville (11). This procedure allows the resolution of three HMW components of outer dynein arms I, II, and V, also referred to as α, β, and γ heavy chains (13) and of at least four HMW components of inner dynein arms III, IV, VI, and VII. Successive Roman numerals were assigned to reflect increasing electrophoretic mobility. The original location of each component in the axoneme was deduced by comparing the molecular compositions of axonemes isolated from wild-type and arm defective mutants (5). Electrophoresis of axonemal proteins in amounts >1 μg does not resolve the same number of components. Small differences in pH of the buffers used for electrophoresis alter the order of migration of II, III, IV, and V (data not shown). Therefore, the identification of HMW components depends on the exact reproduction of the electrophoresis procedure.

The reagents used and respective catalog numbers were as follows. Sodium lauryl sulfate (L-5750), and Tris (T-1503) were both from Sigma Chemical Co., St. Louis, MO. Acrylamide (5521), *N,N'*-methylenebisacrylamide (8383), *N,N,N',N'*-tetramethyl-ethylenediamine, (8178), and ammonium peroxydisulfate (1151) were all from Kodak Laboratory and Specialty Chemicals, Eastman Kodak Co., Rochester, NY. Urea ultra pure was from Schwarz/Mann Biotech, Spring Valley, NY. Sucrose A.C.S. was from Fisher Scientific Co., Pittsburgh, PA. β-Mercaptoethanol came from Aldrich Chemical Co., Milwaukee, WI. Seven stock solutions were prepared as follows: (*1*) 30% acrylamide, 1.2% bisacrylamide; (*2*) 2.12 M Tris-chloride pH 9.35; (*3*) 0.216 M Tris-sulfate pH 6.10; (*4*) 0.82 M Tris-borate pH 8.64, 2% sodium lauryl sulfate; (*5*) 0.2 M EDTA pH 7.5; (*6*) 60% sucrose; (*7*) 10% sodium lauryl sulfate, 4% ethanol. Table I shows the volumes of solution used to prepare cathodic and anodic buffers and a discontinuous gel slab, 30 cm wide, 21 cm long, 0.1 cm thick, formed by a 2.8% acrylamide layer for stacking and a 3.2–5% acrylamide body for the resolution of polypeptides. 33 ml of 3.2% and 31 ml of 5% acrylamide were mixed in a gradient maker while poured into a mold. Isobutanol was overlaid during polymerization and aging of the gel that was performed at room temperature for 16 h. After complete removal of the isobutanol the 2.8% polyacrylamide gel was polymerized 1–3 h before use. 1 μg of $^{35}$S-labeled axonemal polypeptides from a solution containing 2% SDS, 1% β-mercaptoethanol, and 5 mg/ml of protein of specific radioactivity 100,000 cpm/μg were applied to 1.5-cm slots in 40 μl of cathodic buffer and 6% sucrose. Electrophoresis was performed at 20 mA constant current for 24–26 h at room temperature. Detection of polypeptides required 1–4 d autoradiography of dried slab gels.

## *Results*

### *Dynein Arm Components of Dynein Arm-defective Mutants*

At the outset, the mutant *pf* 28, which lacks the outer arms (10), and the mutant *pf* 30, which is partially defective for inner dynein arms (1), were analyzed as were other existing outer and inner arm-defective mutants (5). HMW polypeptides of axonemes were resolved and named following the procedures originally used for dynein arm components of *Chlamydomonas* (19). Fig. 1 shows a portion of the electrophoretograms of wild-type and *pf* 30 axonemal proteins where three major bands, previously identified as outer arm components, are indicated with Roman numerals I, II, and V.

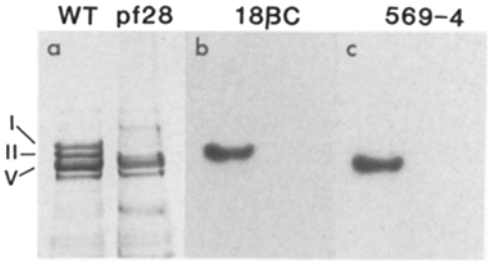


*Figure 2.* Electrophoretograms of axonemal polypeptides of wild-type and mutant *pf* 28 cells and corresponding immunoblots obtained with antibodies specific for outer dynein arm component II or V. (*a*) Silver-stained polypeptides of molecular weight >300,000 resolved by SDS-PAGE. Electrophoresis was performed on the discontinuous gel system similar to that described in Materials and Methods, except that slab gels were of reduced size (6 cm long, 20 cm wide, and 0.075 cm thick). The gel body was formed by 3.2% acrylamide and 10 μg of protein were applied to the slots in 5 μl of cathodic buffer. Outer dynein arm components I, II, and V are indicated in the electrophoretograms of wild-type polypeptides. Two sets of the same samples were subjected to electrophoresis in parallel and then transferred to nitrocellulose. (*b*) Autoradiogram of the nitrocellulose incubated with 18βC, a monoclonal antibody specific for component II. (*c*) Autoradiogram of the nitrocellulose incubated with 569-4, a monoclonal antibody specific for component V.

These components are absent in the electrophoretogram of *pf* 28 axonemal proteins. In contrast, inner arm components III and IV are present in the electrophoretograms of wild-type and *pf* 28 proteins but absent in that of *pf* 30. Arm components VI, VII, and VIII are present in every electrophoretogram and each is partially resolved as two bands. The apparent molecular weight of each of these components was calculated in a previous study and is >300,000 (19). The estimated molecular weight of components III and IV was 325,000 and 315,000, respectively. Two polypeptides, components IX and X, are absent in these preparations of axonemes and were usually resolved from the other polypeptide HMWs in electrophoretograms of axonemal polypeptides containing outer dynein arm components (19). Component X was considered to be a subunit of outer arm dynein (5) and could be a proteolytic fragment derived from component I (13).

Wild-type and *pf* 28 axonemal polypeptides were also analyzed with sets of monoclonal antibodies specific for polypeptides II or V. Immunoblots indicate that polypeptides II and V are present in axonemes of wild-type cells but absent in the axonemes of the mutant *pf* 28. Fig. 2, *b* and *c* shows the immunoblots incubated with the antibody 18βC, specific for component II, and 569-4, specific for component V, respectively. No antibody binding to *pf* 28 polypeptides is detected although the binding to wild-type polypeptides is extensive. The same result was obtained with other monoclonal antibodies specific for different epitopes of II and V (not shown). Therefore the mutation of *pf* 28 does not cause partial assembly of components II and V to the axoneme or alter their electrophoretic mobility.

These observations confirm the results of experiments that were performed by different electrophoretic procedures (1, 10). The axoneme of the mutant *pf* 28 lacks all HMW components of outer dynein arms and the axoneme of *pf* 30 lacks only two HMW polypeptides, components III and IV of inner dynein arms. Therefore, separations of ATPase activities located in the inner dynein arm of *pf* 28 axonemes can be performed without interference from outer dynein arm ATPases. Previous analysis showed that components VI and VII copurify with two different ATPase activities (20). However, the function of components III and IV has not yet been identified and is the subject of this investigation.

*Table II. Salt Extraction of Axonemal $Mg^{++}$-activated ATPases*

| | Total protein | Protein extracted | | Total ATPase | ATPase extracted | |
|---|---|---|---|---|---|---|
| | *mg* | *mg* | *%* | *U* | *U* | *%* |
| Wild type | 1.87 | 0.49 | 26 | 0.80 | 0.61 | 76 |
| *pf* 28 | 10.00 | 2.70 | 27 | 0.32 | 0.14 | 44 |
| *pf* 30 | 2.11 | 0.58 | 28 | 0.99 | 0.69 | 70 |

The unit of ATPase activity was defined as the amount of enzyme that hydrolizes 1 μmol of Pi per minute. Chromatograms of these preparations of extracted proteins are shown in Fig. 3.

### *Chromatography of Polypeptides from Dynein Arms*

Electrophoretic and biochemical analyses of chromatographic fractions of *pf* 28 axonemal proteins suggested that a new ATPase activity copurifies with components III and IV. This activity was extracted from the axonemes in the presence of 0.5 M NaCl, 1 mM ATP, 4 mM $MgCl_2$, 1 mM dithiothreitol, and 10 mM Hepes (pH 7.2), and was separated by chromatography on hydroxyapatite from the inner dynein arm ATPases composed of polypeptides VI and VII as previously described (20).

Percentages of proteins and $Mg^{++}$-activated ATPase activities extracted from the axonemes of wild-type *pf* 28 and *pf* 30 are reported in Table II. 39–68% (average was 49 ± 11%) of ATPase activity was extracted from four different preparations of *pf* 28 axonemes and 18–25% (average was 21 ± 3%) remained in the residue. Evidence showing that all HMW components of *pf* 28 axonemes are extracted to the same extent will be described in the second part of this section.

Chromatograms of proteins and $Mg^{++}$-dependent ATPase activities, present in salt extracts of wild-type *pf* 28 and *pf* 30 axonemes, are shown in Fig. 3. Although the salt extract of *pf* 28 axonemes contained approximately five times the amount of protein of the other two extracts, protein and activity elution profiles of each chromatogram are similar in the 0.05–0.15 M sodium phosphate range used for elution. However, the profiles obtained with the *pf* 28 extract (Fig. 3 *b*) differ from the others in the range 0.15–0.25 M of sodium phosphate. A major peak of protein and ATPase activity eluted at 0.18 M Na phosphate is present in the chromatogram of wild-type and *pf* 30 proteins (Fig. 3, *a* and *c*) but absent in the chromatogram of *pf* 28 proteins (Fig. 3 *b*). These chromatographic separations were interpreted on the basis of previous analyses (20). In fact, the two inner dynein arm ATPases associated with subunits VII and VI are eluted between 0.05–0.12 M sodium phosphate and outer dynein arm ATPases and their subunits I, II, and V form the peak eluted at 0.18 M sodium phosphate. Electrophoretic analysis of HMW polypeptides of wild-type axonemes showed that components I, II, III, IV, and V are not resolved by chromatography in these conditions (20).

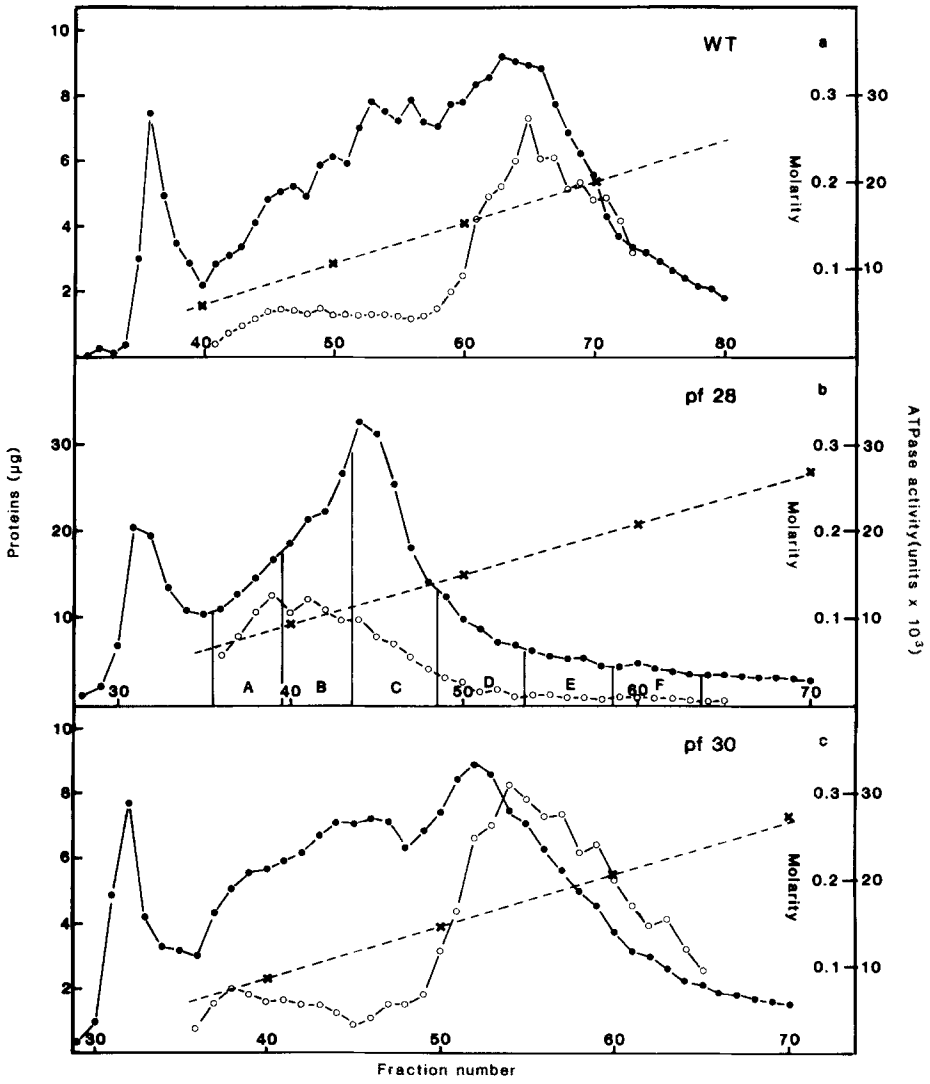


*Figure 3.* Chromatograms of NaCl-extracted proteins from wild-type and mutant axonemes. $^{35}$S-labeled proteins (~100,000 cpm/μg) were loaded on a 0.5 × 4 cm column of hydroxyapatite and eluted with 6 ml gradient of sodium phosphate buffer, pH 6.8, from 0 and 0.4 M in the presence of 0.5 M NaCl. (●) Proteins; (○) ATPase activity; (×) buffer molarity. (*a*) Chromatogram of wild-type proteins. (*b*) Chromatogram of *pf* 28 proteins. (*c*) Chromatogram of *pf* 30 proteins. *A–F* in *b* designate the pools of chromatographic fractions that were prepared.

Pools designated A–F from the chromatogram of *pf* 28 axonemal proteins were made with fractions eluted in the 0.07–0.23 M sodium phosphate range. Polypeptides contained in pooled chromatographic fractions were analyzed by gel electrophoresis, enzymatic assays, and sedimentation on sucrose gradient after a dialysis against 5 mM TrisCl, pH 8.3, 0.1% NP-40, and 0.1 mM phenylmethylsulfonyl fluoride in the presence or absence of 0.2 mM EDTA, 0.2 mM EGTA. Fig. 4 shows the electrophoretograms of HMW components from a chromatogram similar to that shown in Fig. 3 *b*. Components III and IV are present in pools C, D, E, and F and are partially purified from components VI, VII, and VIII. These last are enriched in pools A, B, and C and each is partially or completely resolved in two bands, as seen in Fig. 1. Electrophoresis of polypeptide fractions from chromatograms of wild-type salt extracts confirmed that components III and IV are eluted between 0.15–0.25 M sodium phosphate, as are the outer dynein arm components I, II, and V (not shown).

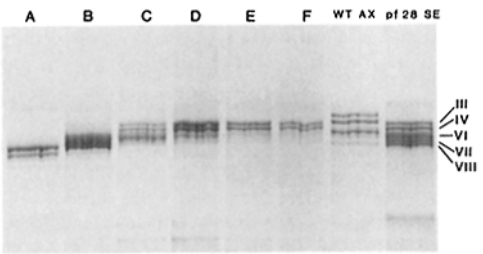


*Figure 4.* Electrophoretograms of $^{35}$S-labeled polypeptides contained in pools A–F, in wild-type axonemes, and in salt extracts from *pf* 28 axonemes. The autoradiogram shows polypeptides of molecular weight >300,000. The load of radiolabeled proteins was 50,000 cpm for pools A–F and 100,000 cpm for axonemes and salt extract. Dynein arm components III, IV, VI, VII, and VIII are indicated in the electrophoretogram of polypeptides of the salt extract from *pf* 28 axonemes.

Fig. 4 (last two lanes) also shows the electrophoretograms obtained with protein samples of equal mass of wild-type axonemes and *pf* 28 salt extract. Bands of components III, IV, VI, VII, and VIII of *pf* 28 salt extract are as intense as the bands formed by outer arm components I, II, and V. Therefore, there was enrichment of each HMW component in *pf* 28 salt extract (see Fig. 1 for comparison) and each HMW component appears to be extracted to the same extent. Salt-insoluble residues of *pf* 28 axonemes contained similar amounts of each HMW component at the trace level (not shown).

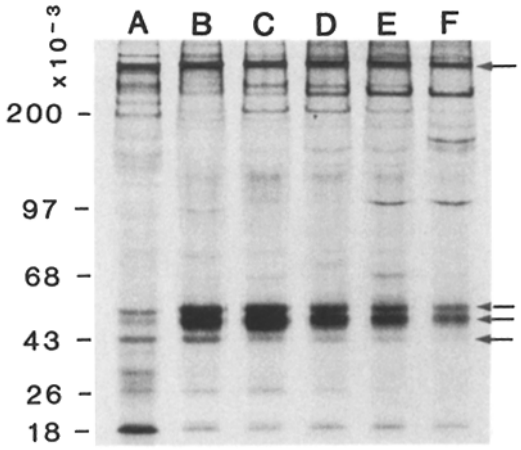


*Figure 5.* Electrophoretograms of all $^{35}$S-labeled polypeptides contained in pools *A–F*, as they are resolved in a 4–11% polyacrylamide gradient gel of reduced size (see legend to Fig. 2). The load of radiolabeled proteins was 3,000 cpm for each sample. HMW components, tubulin subunits, and actin shown in the autoradiogram of polypeptides are indicated by arrows. The identification of these components was based on their electrophoretic mobility. The position of molecular weight standards is indicated on the left.

Fig. 5 shows the electrophoretograms of all polypeptides present in pools A–F. Each pool contains HMW dynein components, tubulin subunits, a 42,000-mol-wt polypeptide (Fig. 5, *arrows*), as well as a discrete number of other polypeptides.

ATPase activities of pools A–F were assayed in different conditions and the corresponding specific activities are reported in Table III. Specific ATPase activities of each pool are similar in different conditions. However, specific ATPase activities of pools A and B, which contain the highest concentrations of components VII and VI, are approximately two times higher than the specific activities of pools E and F, which contain only trace amounts of these components. Therefore, one or more previously unknown ATPase activities may be present in pools E and F. Components III and IV are good candidates for subunits of the ATPase since they are present in pools E and F at high concentration and in a relatively pure state. GTPase activity of pools D and F, measured at pH 7.2 in the presence of $Mg^{++}$ and EGTA, respectively, was 2.3 and 3.9% of the ATPase activity. Therefore, the activity present in pools D and F appears to be specific for ATP, similar to all other dynein arm ATPases (19, 20).

*Table III. Specific ATPase Activities of Chromatographic Fractions of pf28 Axonemal Proteins*

| Pool | pH 8.8, $Mg^{++}$ | pH 7.2 $Mg^{++}$, EGTA | pH 7.2, $Mg^{++}$, $Ca^{++}$ |
|---|---|---|---|
| A | 1.11 | 1.03 | 1.16 |
| B | 0.89 | 0.82 | 0.86 |
| C | 0.67 | 0.54 | 0.66 |
| D | 0.68 | 0.58 | 0.75 |
| E | 0.50 | 0.52 | 0.58 |
| F | 0.51 | 0.42 | 0.58 |

Specific ATPase activity is expressed in $\mu mol \cdot min^{-1} \cdot mg^{-1}$. Average values obtained in duplicate assays are reported.

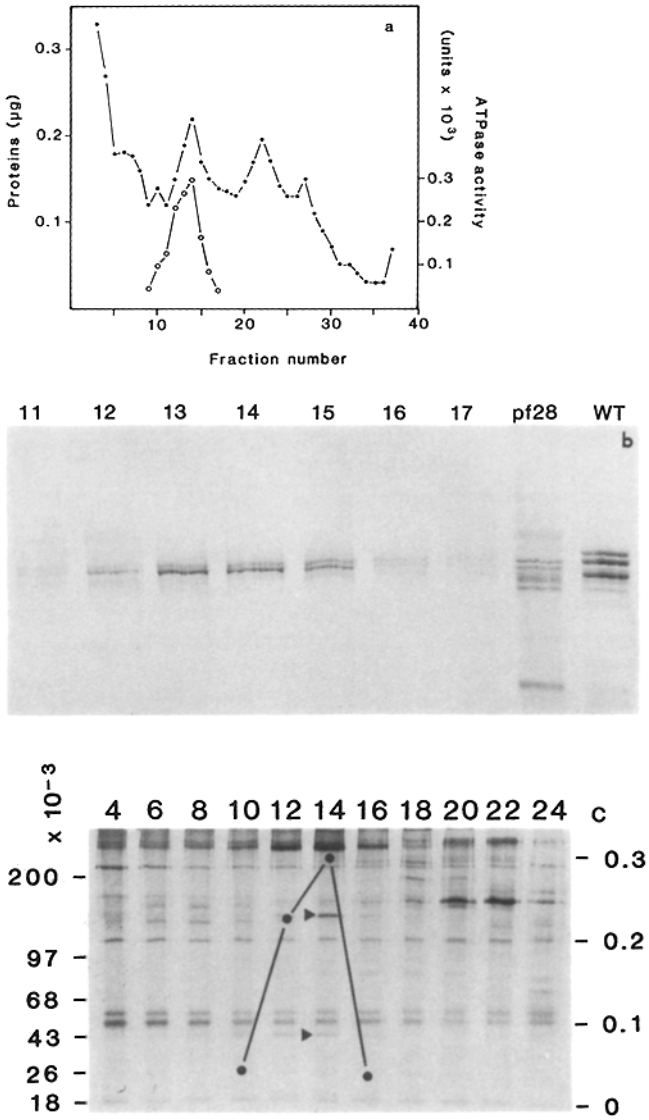


*Figure 6.* Sucrose gradient sedimentation and electrophoretograms of polypeptides. (*a*) Sedimentation profile of ~4.5 µg of protein (113,000 cpm/µg) contained in pool F. Centrifugation of a 5–20% sucrose gradient in 0.1% NP-40, 0.2 mM EDTA, 0.2 mM EGTA, 5 mM Tris-Cl (pH 8.3) was performed in a rotor (model SW55; Beckman Instruments, Inc., Palo Alto, CA) at 44,000 rpm for 10 h at 5°C. $^{125}$I-Catalase labeled by the chloramine T method was sedimented as standard in a parallel gradient. Recovery of protein was 126% and recovery of ATPase activity in the peak of activity was 32%. Direction of sedimentation was from right to left. (●) Proteins; (○) ATPase activity. (*b*) Electrophoretograms of polypeptides of molecular weight >300,000 contained in sucrose gradient fractions 11–17, in *pf* 28, and in wild-type axonemes. Slots were loaded with 40-µl aliquots of the fractions or with 40 µl of 15% sucrose (from the 20% stock solution used for the sucrose gradient) containing 50,000 cpm of axonemal proteins. (*c*) Electrophoretograms of all polypeptides contained in even fractions 4–24 of the sucrose gradient. Electrophoretic conditions are as in the experiment shown in Fig. 5. Slots were loaded with 10 µl from each fraction. Superimposed on fractions 10–16 are reported values of ATPase activity expressed in units × $10^3$ as in *a*. Triangles indicate polypeptides of molecular weight 140,000 and 42,000 that cosediment with the activity. The position of molecular weight standards is indicated on the left.

### *Partial Purification of a New ATPase*

The identification of a new ATPase was achieved after those proteins contained in chromatographic fractions of *pf* 28 salt extract were sedimented on a sucrose gradient. Fig. 6 *a* shows the sedimentation profiles of proteins and ATPase activity of a pool prepared as pool F was. The peak of activity has a sedimentation coefficient of 11S. Specific ATPase activity at the peak is 1.4 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ at pH 7.2 in the presence of both $Mg^{++}$ and $Ca^{++}$. Additional experiments performed with pool F proteins indicated that no peak of ATPase activity other than the 11S peak is present in the gradient (not shown). Fig. 6 *b* shows the electrophoretograms of HMW polypeptides contained in fractions *11–17* of the sucrose gradient. Components III and IV are prevalent in these fractions and are isolated from other HMW components in fraction *14*. Their distribution approximately coincides with that of protein and ATPase activity, although they are present in different concentration in fractions *12–16*. Component VI in fraction *15* and component VII in fraction *12* are present in trace amounts. Other sedimentations were performed with pool E and pool D proteins, which contain higher concentrations of components VI and VII. These experiments confirmed that components III and IV sediment with a coefficient of 11S and are partially separated from components VI and VII, which sediment with coefficients of 10–11 and 12.5S, respectively (not shown). Isolated HMW components III and IV were also obtained from pool E proteins, and specific ATPase activity up to 2.3 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ was found at the 11S peak of sedimentation.

Electrophoretograms of all polypeptides present in even fractions 4–24 of the sucrose gradient (Fig. 6 *c*) indicate that fractions *12–16* contain two polypeptides of molecular weight 140,000 and 42,000 cosedimenting with the peak of activity and polypeptide III and IV. Although the 140,000-mol-wt polypeptide is present at the highest concentration in fraction *14*, the 42,000-mol-wt polypeptide is present in similar concentrations in fractions *12* and *14*. Therefore, the new inner dynein arm ATPase activity copurifies with components III and IV, the 140,000- and 42,000-mol-wt polypeptides, but the peaks of sedimentation of these components do not coincide. Fractions *10–16* also contain tubulin subunits and other unidentified polypeptides.

### *One Component of the 11S Complex Is Actin*

The 42,000-mol-wt polypeptide cosedimenting with the 140,000-mol-wt polypeptide and components III and IV was identified as actin on the basis of its electrophoretic properties. Fig. 7 shows a portion of a two-dimensional map resolving α, β tubulin subunits and the 42,000-mol-wt polypeptide contained in the 11S sedimentation peak. The lowest component migrates as actin. An extensive characterization of this polypeptide was reported elsewhere (18). No other axonemal component has the same electrophoretic behavior in two-dimensional gel electrophoresis.

## *Discussion*

A previously unknown ATPase has been identified as a component of a protein fraction extracted from the axoneme of the mutant *pf* 28. Partial purification of the molecular complex carrying out the ATPase activity was achieved with a three-

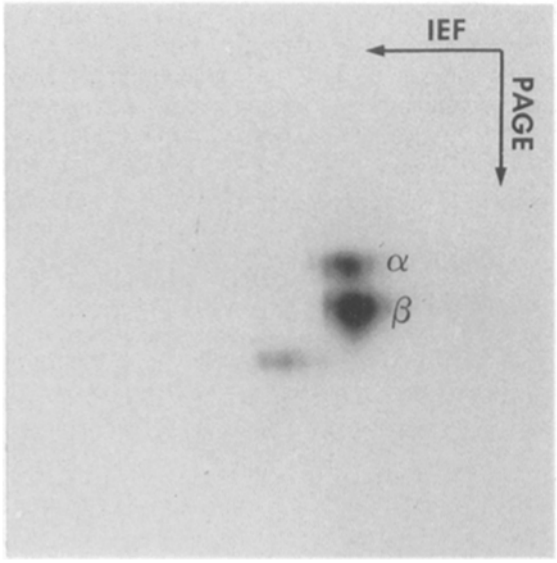


*Figure 7.* Two-dimensional map of polypeptides cosedimenting with components III and IV and the 140,000-mol-wt polypeptide. The polypeptides were applied at the anode of the gel used for isoelectric focusing and detected by autoradiography. A portion of the original map is shown. The two major components are α and β tubulin subunits.

step procedure consisting of preferential extraction of dynein arms from the axonemes, chromatography on hydroxyapatite in the presence of 0.5 M NaCl, and sedimentation on a sucrose gradient. In the past this approach led to the purification of two inner dynein arm ATPases from axonemes of wild-type cells. The application of the same procedure to axonemes of *pf* 28, a mutant lacking outer dynein arms, has allowed the isolation of a third ATPase, which is likely located in the inner dynein arms.

The new ATPase differs from previously identified inner dynein arm ATPases in its chromatographic and sedimentation properties, molecular composition, and specific activity. In fact, the activity is carried out by a complex comprising component III and/or IV, the polypeptide of 140,000 mol wt, and actin. Fractions containing the ATPase in its highest specific activities did not include any subunit of known inner dynein arm ATPases except actin. Other molecules present in the active fractions do not form a peak of sedimentation coincident with the ATPase activity. Whether the HMW components could hydrolyze ATP in the absence of the 140,000-mol-wt polypeptide and actin was not determined.

The sedimentation coefficient of the complex carrying the activity is 11S. This is a reasonable value for a protein of 400,000 mol wt and is close to those of the inner dynein arm ATPases, which are comprised between 10 and 12.5S. However, the 11S complex appears to contain two HMW components instead of one, as the 10–11 and 12.5S complex. Therefore three complexes have similar sedimentation behavior and apparently very different molecular weight. This paradox is explained if HMW component III and IV are different electrophoretic forms of the same polypeptide. Alternatively, two complexes containing HMW component III or IV have similar sedimentation coefficient. Hints in support of this last explanation are found in Fig. 6, *a*, *b*, and *c*. The peak formed by the ATPase activity is skewed toward the denser

part of the gradient. The concentration of component IV in the peak of activity is higher than that of component III, although these components apparently were equimolar before sedimentation. Finally, the actin component of the complex appears to be enriched in fractions sedimenting with coefficient above 11S. All these facts suggest that two complexes with coefficients close to 11S were isolated with our procedure. However they do not imply that both complexes are active ATPases.

Goodenough et al. (4) have also used the mutant *pf* 28 for the characterization of inner arm dynein subunits. They analyzed various complexes by procedures that differ from those described here and reported the resolution of two species sedimenting in sucrose gradient with sedimentation coefficients of 17.7 and 12.7S. The 12.7S sedimentation peak contained two HMW components and at least four intermediate and low molecular weight polypeptides. The 17.7S complex was formed by two HMW components, called III and IV, and a polypeptide of molecular weight 140,000. Actin was not found in the 17.7S complex. Assuming that the HMW components III and IV identified here are identical to those indicated by Goodenough et al. (4), then the 17.7S particle is an intermediate product of dissociation of an inner dynein arm subunit. This product probably is further dissociated into the two different 11S complexes that were mentioned above. A similar pattern of dissociation from an 18 into a 13.1 and a 14.5S particle was found to occur with one subunit of *Chlamydomonas* outer arm dynein (13). A control of the identity of the 17.7S complex could be performed with the axonemes of the mutant *pf* 30 which are missing the HMW components III and IV.

Several lines of evidence indicate that the subunits of the new ATPase are inner dynein arm components. First, the same polypeptides or a subset of them appear to be deficient in axonemes of two mutants, *pf* 23 and *pf* 30. Electron microscopic analysis revealed that both mutants are deficient but not completely depleted of inner dynein arms, *pf* 23 being more defective than *pf* 30 (1, 5). The deficiency of ~75% of the inner arms from the axonemes of *pf* 23 was correlated with the deficiency of 11 polypeptides, including components III and IV, the polypeptide of molecular weight 140,000 and actin. The lesser degree of deficiency of *pf* 30 has been correlated with the absence of III and IV, and the 140,000 polypeptide, components that copurify with the new ATPase. Therefore, this ATPase may derive from a substructure or a differentiated form of inner dynein arms (1, 3). Second, the four polypeptides analyzed in this study are extracted from the axonemes, as the subunits of all other dynein arm ATPases, in a process that is selective for dynein arms. In fact, 20 putative outer and inner dynein arm components, together with ~30 other polypeptides, are extracted from wild-type axonemes in conditions causing the disassembly of nearly the totality of outer and inner dynein arms. In contrast, >200 other axonemal polypeptides and substructures like radial spokes, nexin links, and one of the central microtubule pair are remaining preferentially in the insoluble residue (19). Third, the new ATPase activity consists of a complex containing at least one polypeptide of molecular weight >300,000 (HMW), which has a distinctive electrophoretic behavior. These HMW polypeptides, as all the HMW subunits of ATPases located in the dynein arms, migrate in a polyacrylamide gel with similar and exceptionally low mobility. Fourth, the ATPase copurifies with actin as the two other inner dynein ATPases previously characterized (20).

*Table IV. Match of ATPase Subunits and Polypeptides Deficient in pf23 Axonemes**

| Polypeptides deficient in *pf* 23 | Apparent molecular weight | ‡11S ATPase | 10–11S ATPase | 12.5S ATPase |
|---|---|---|---|---|
| III | 325,000 | + | − | − |
| IV | 315,000 | + | − | − |
| VI | 315,000 | − | + | − |
| VII | 310,000 | − | − | + |
| VIII | 310,000 | − | − | − |
| 1′ | 110,000 | − | − | − |
| 2′ | 83,000 | − | − | − |
| 3′ | 42,000 | + | + | + |
| 4′ | 28,000 | − | + | + |
| 5′ | 28,000 | − | + | + |

* Criteria used for the denomination of polypeptides and the determination of their apparent molecular weight were as described (5). The identification of subunits of each ATPase with components deficient in the axoneme of the mutant *pf* 23 was based on electrophoretic properties of each polypeptide in one- and two-dimensional gel electrophoresis.

‡ The sedimentation peak of the 11S ATPase also contains a 140,000 subunit that is not reported in this table. The deficiency of this polypeptide in the axoneme of *pf* 23 has been found by B. Huang (personal communication) and Goodenough et al. (4). The 140,000-mol-wt polypeptide and the polypeptide designated 1′ were also deficient is the axoneme of *pf* 30 (Huang, B., personal communication).

Actin has been found in each complex that has ATPase activity and derives from the dissociation of inner dynein arms. It is present in the 11S ATPase in spite of the fact that the active molecule has chromatographic properties that are different from those of the other two inner dynein arm ATPases, and that HMW components III and IV are not phosphorylated like components VI, VII, and VIII (20). Possible structural or functional roles of the actin in the inner dynein arm ATPases have already been discussed (20, 21). Finding actin in a third inner dynein arm ATPase only suggests that inner dynein arm substructures are similar. Extensive information concerning axonemal actin, its structural form, its stoichiometry in each inner dynein arm ATPase, and its location relative to the subunit carrying the site of ATP hydrolysis will be needed in order to understand its role in the movement of the inner dynein arms.

Additional inner dynein arm ATPases may exist. Table IV shows that not all inner dynein arm components, as they are defined for their deficiency in the axonemes of the mutant *pf* 23, were isolated in the form of an ATPase. HMW component VIII and component 1′ and 2′ have not been characterized after extraction and isolation. In addition, polypeptides VI, VII, and VIII are resolved in two components in several electrophoretograms shown in this study. This effect may be caused by differential phosphorylation of the polypeptides or proteolysis. Alternatively, the splitting of electrophoretic bands indicates that more than five HMW components of inner dynein arms exist in the axonemes. The isolation of mutants defective for the phosphorylation of HMW components and of mutants, like *pf* 30, that are missing subsets of the inner dynein arm polypeptides could be very useful for the definition of this point.

The multiplicity of inner dynein arm ATPases indicates that inner dynein arms are differentiated at the level of their

substructures. Assuming that each substructure contributes to the sliding of adjacent outer double microtubules, differences among these substructures create possible sites for the modulation of localized movements of the axoneme. The modulation itself is probably changed by mechanisms depending on phosphorylation and/or the presence of $Ca^{++}$ ions, as in other moving structures. Therefore the substructures of inner dynein arms could be envisioned as slightly different axonemal "motors" endowed with very complex systems of regulation. Comprehensive studies of inner and outer dynein arms eventually will explain the molecular mechanisms generating and controlling the movements of the axonemes. In short, they provide models for the analysis of molecules that generate movement in other systems of microtubules.

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