Identity and Origin of the ATPase Activity Associated with Neuronal Microtubules. II. Identification of a 50,000-Dalton Polypeptide with ATPase Activity Similar to F-1 ATPase from Mitochondria

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ABSTRACT We determined that the ATPase activity contained in preparations of neuronal microtubules is associated with a 50,000-dalton polypeptide by four different methods: (a) photoaffinity labeling of the pelletable ATPase fraction with [γ-^{32}P]-8-azido-ATP; (b) analysis of two-dimensional gels (native gel $\times$ SDS slab gel) of an ATPase fraction solubilized by treatment with dichloromethane; (c) ATPase purification by glycerol gradient sedimentation and gel filtration chromatography of a solvent-released ATPase fraction, (d) demonstration of the binding of affinity-purified antibody to the 50-kdalton polypeptide to ATPase activity in vitro. Beginning with preparations of microtubules we have purified the ATPase activity greater than 700-fold and estimate that the purified enzyme has a specific activity of 20 μmol $P_i \cdot mg^{-1} \cdot min^{-1}$ and comprises 80–90% of the total ATPase activity associated with neuronal microtubules.

With affinity-purified antibody we also demonstrate cross-reactivity to the 50-kdalton subunits of mitochondrial F-1 ATPase and show that the antibody specifically labels mitochondria in PtK-2 cells. Biochemical comparisons of the enzymes reveal similar but not identical subunit composition and sensitivity to mitochondrial ATPase inhibitors. These studies indicate that the principal ATPase activity associated with microtubules is not contained in high molecular weight proteins such as dynein or MAPs and support the hypothesis that the 50-kdalton ATPase is a membrane protein and may be derived from mitochondria or membrane vesicles with F-1-like ATPase activity.

In the preceding paper (21) we reported that the ATPase activity contained in preparations of neuronal microtubules is particulate and that as much as 90–95% may be associated with contaminating membrane vesicles that copurify with microtubules during cycles of in vitro purification. We also established that soluble, column-purified fractions of microtubule-associated proteins (MAP-1, MAP-2, and tau factors) and another high molecular weight protein do not contain ATPase activity. However, the association of ATPase with membranes was not directly demonstrated, and the molecular identity of ATPase was not established. Using conventional biochemical purification techniques, we could achieve only a 50-fold enrichment in ATPase activity, which did not allow identification of the subunits involved. We therefore employed two new methods, including a photoaffinity-labeling procedure with [γ-^{32}P]-8-

azido-ATP and a solvent extraction procedure that enabled us to identify and then isolate and partially purify the enzyme greater than 700-fold. We discovered the ATPase to be contained in a 50,000-dalton subunit and estimated the specific activity of the purified enzyme to be 20 μmol $P_i \cdot mg^{-1} \cdot min^{-1}$, indicating that it is present in microtubule preparations as a trace component at 0.05%.

To confirm the identity of the ATPase as the 50-kdalton subunit and to determine its origin, we isolated and affinity-purified antibodies from rabbits inoculated with purified 50-kdalton protein. The antibody bound to native ATPase activity in vitro and cross-reacted with the F-1 ATPase subunits from mitochondria. In this report we compare some of the physical and biochemical properties of the 50-kdalton brain microtubule ATPase and mitochondrial F-1 ATPase, and note that despite

many similarities there are also significant differences regarding subunit composition and sensitivity to the mitochondrial ATPase inhibitor oligomycin.

These observations confirm the idea that the ATPase activity in preparations of microtubule protein is associated with membrane components and do not support our earlier hypothesis that cytoplasmic microtubules are associated with dyneinlike ATPase. To the extent that microtubules purified by in vitro assembly contain the full complement of proteins associated with microtubule function in vivo, these observations also suggest that the mechanism for organelle movements may be based on other microtubule- or membrane-associated components.

MATERIALS AND METHODS
Preparation of ATPase Fractions

PELLETABLE FRACTION OF ATPASE: Microtubule protein purified by one or two cycles of in vitro assembly-disassembly as described in the preceding paper (21) was resuspended in 0.1 M PIPES buffer and centrifuged (200,000 g, 60 min, 5°C). The resulting cold pellets were resuspended in 30 mM Tris-SO$_4$, pH 7.7, containing 1.0 mM EDTA and DTT (TED), sedimented as above to remove remaining soluble proteins, resuspended in TED, and stirred overnight to allow dissociation of adherent proteins including actin. The extracted material was pelleted as above, frozen in liquid nitrogen, and stored at −80°C. This fraction, which was rich in membrane vesicles, contained 80–95% of the initial units of ATPase activity present in samples of in-vitro-purified microtubules.

DETERGENT-SOLUBLE FRACTION OF ATPASE: Aliquots of the pelletable ATPase described above were resuspended in 0.1 M PIPES containing 0.1% Triton X-100 or 100 mM octylglucoside with a glass-Teflon homogenizer, incubated at 37°C for 30 min and centrifuged (150,000 g, 60 min, 5°C). The resulting supernate contained 70–90% of the remaining ATPase activity as estimated by the dilution procedure described in the preceding paper (21). Since detergent-extracted ATPase was observed to be partially inactivated by freezing, enzyme was prepared fresh each time before use.

DICHLOROMETHANE-RELEASED ATPASE: Preparations of dichloromethane-released ATPase were made by the procedure described by Beechey et al. (5) as modified by Apps (4) for isolating F-1 ATPase from chromaffin granule membranes. The pelletable fraction of ATPase (10 mg) was resuspended in TED with a glass-teflon homogenizer to 5 mg/ml and brought to 22°C in a glass centrifuge tube. Four volumes of dichloromethane were added and the mixture immediately vortexed for 20 s. The emulsion was broken by centrifugation (30,000 g, 20 min, 22°C), and the top aqueous layer containing ATPase was removed and centrifuged to eliminate remaining particulate material (100,000 g, 30 min, 25°C). The ATPase activity in the resulting supernate (dichloromethane-released ATPase) was stable at 22°C for 2–3 d but was highly labile at low temperature, decaying with a half-life of 5 min at 5°C.

PAGE

SDS slab gels containing 12% acrylamide were prepared by the method of Laemmli (18) except that 0.4% DATD was used as the cross-linker (1). SDS tube gels containing 5% acrylamide and 0.1 M sodium phosphate were prepared by the method of Shapiro et al. (28) as described by Weber and Osborn (35). Gels were stained with Coomassie Blue and destained as described by Fairbanks et al. (11).

Native gels containing 5% acrylamide, 750 mM Tris-Cl, pH 8.9, and 0.25 M sucrose were prepared by the method of Knowles and Penefsky (17). Dichloromethane extract (0.1 ml) was applied to the gel and electrophoresis was performed at 1.5 ma/gel for 4 h at 22°C. Gels were stained for protein with Coomassie Blue and for ATPase activity in 30 mM Tris-NO$_3$, pH 8.0, containing 5 mM Mg(NO$_3$)$_2$, 1 mM Pb(NO$_3$)$_2$, and 3 mM ATP according to the procedure of Wachstein and Meisel (34). After maximal development of the lead phosphate band (1–2 h), gels were rinsed and stored in distilled water. For photography, thoroughly rinsed gels were placed in 10 mM ammonium sulfide to produce black insoluble lead sulfide. The stained gels were rinsed and stored in distilled water.

Two-dimensional gels were prepared by a procedure similar to that used for preparing two-dimensional gels by the method of O'Farrell. Native tube gels were soaked in a solution of 10% SDS and 10% mercaptoethanol for 10 min and laid on top of a standard Laemmli SDS slab gel. During electrophoresis, the proteins leave the cylindrical gel and enter the SDS slab gel, thereby forming a two-dimensional "map" of polypeptides contained in the sample. The proteins associated with ATPase activity can be readily identified once the electrophoretic mobility of the ATPase activity on the native gel has been determined.

Determination of Protein Concentration and ATPase Activity

Protein concentration was determined by the method of Lowry et al. (19) and by the method of Bradford (7) using bovine serum albumin as a standard, ATPase activity was determined as described in the preceding paper (21). The amount of inorganic phosphate released by ATP hydrolysis was determined by the colorimetric procedure of Pollard and Korn (24).

Summary of Antibody Purification Procedures

Dichloromethane extract (300 mg) was fractionated on glycerol gradients, labeled with dansyl chloride (29), fractionated by electrophoresis on preparative Laemmli gels, and the fluorescent 50-kdalton bands were excised and the protein was eluted (4.7 mg) by the method of Stephens (29). The eluted protein was adsorbed onto fine particles of Bentonite by the method of Gallily and Garvey (12), emulsified with Freund's adjuvant, and a total of 1,770 μg of 50-kdalton protein was injected into each of three New Zealand white rabbits over a period of 3 mo. Antiserum was examined for specific antibody by a solid-phase-binding assay using the procedure of Tsu and Herzenberg (32) as modified by Kiehart (personal communication). We used polystyrene microtiter plates and ^{125}I-labeled protein A to quantitate the amount of antibody binding. The specificity of antibody fractions was determined by examining the binding of antibody to proteins that were fractionated by SDS gel electrophoresis and transferred by electrophoretic blotting onto nitrocellulose sheets by the method of Towbin et al. (31). Strips labeled with ^{125}I-protein A were dried, and autoradiograms were prepared to identify immunoreactive components. To purify 50-kdalton-specific antibody, an affinity column containing 50-kdalton protein was prepared from cyanogen bromide-activated Sepharose by standard methods (25), and specific antibody was eluted using a modification of the procedure described by Bennett and Stenbuck (6), which gave a 100-fold purification of specific antibody. Alternatively, we purified antibody by a modification of the procedure described by Newman et al. (22) using Towbin's immunoblotting procedure to transfer proteins onto nitrocellulose paper. This method gave a 1,000-fold purification of specific antibody.

Examination of the Binding of ATPase Activity to Beads Containing Protein A-Sepharose and Affinity-purified Antibody

Affinity-purified antibody and control antibody preparations (the flow-through fraction from the affinity column and a fraction of IgG from preimmune serum) were suspended in 10 ml of PBS-0.1% Triton (0.4 mg/ml) and mixed overnight with 0.5 ml of protein A-Sepharose CL 4B (Pharmacia Inc., Piscataway, NJ). Examination of the supernate from this mixture by the solid-phase binding assay and SDS gel electrophoresis revealed that >90% of the IgG bound to the protein A-Sepharose beads. The Sepharose beads were washed three times by centrifugation (500 g, 30 s in a clinical centrifuge) and resuspension in 10 ml of 30 mM Tris-HCl, pH 8.0 with 0.1 mM EDTA and 1.2 mM MgCl$_2$ to remove unbound protein, transferred to a 1.5-ml Eppendorf tube, resuspended in buffer to a volume of 1.4 ml containing 0.5 ml of slurry, 0.1% Triton X-100 and 20 μg/ml dichloromethane extract and placed on a motor-driven rotator at 22°C. After 5 h, the slurry was pelleted and 0.2 ml of the supernate was removed and assayed for ATPase activity. The Sepharose slurry was rinsed by three cycles of centrifugation and resuspension and resuspended to 1.4 ml with buffer. A 0.2-ml aliquot of the slurry was removed and assayed for ATPase activity.

Biological Materials and Chemical Reagents

Rat liver mitochondria prepared by the method of Schneider and Hogeboom (27) and mitochondrial F-1 ATPase prepared by the method of Catterall and Pedersen (9) were kind gifts from the laboratory of Dr. Peter L. Pedersen. Tris, ultrapure grade, was obtained from Schwarz/Mann, Inc. (Orangeburg, NY). PIPES, sodium salt was obtained from Calbiochem-Behring Corp. (San Diego, CA). All other biochemical materials and nucleotides were obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Photoaffinity Labeling of ATP-binding Polypeptides

In collaboration with Dr. Boyd Haley (University of Wyoming, Laramie, WY) we used a photoaffinity analogue of ATP,

[γ-³²P]-8-azido-ATP, to identify the ATP-binding subunits of the brain microtubule ATPase using a combination of photoaffinity labeling, SDS gel electrophoresis, and gel autoradiography. The procedures used for synthesizing 8-azido-ATP and for obtaining specific photolabeling of protein kinase have been described previously by Haley and colleagues (10, 13, 14, 15). A report on the use of this analogue to identify ATP-binding subunits of flagellar dynein and mitochondrial F-1 ATPase will be presented elsewhere.

In control experiments we demonstrated that the pelletable fraction of microtubule ATPase utilized 110% 8-azido-ATP as well as ATP, and that the enzyme has a K_m of ~12 μM at 0°C in the buffer used to assay ATPase activity. In addition, photolysis with 8-azido-ATP inhibited ATPase activity by 30%, whereas exposure to UV light alone or to 8-azido-ATP without photolysis had little or no effect on ATPase activity. Thus, 8-azido-ATP is utilized as efficiently as ATP by the ATPase and photolabeling in the presence of analogue appeared to block one-third of the enzymatic sites in the preparation.

Preparations of brain extract and the pelletable ATPase fraction were mixed with 20 μM 8-azido-ATP, photolyzed for 1 min as described in the legend to Fig. 1, and the photolabeled proteins were identified by electrophoresis and autoradiography. To distinguish those proteins that were specifically photolabeled from those that were simply phosphorylated by protein kinase activity, we also examined samples that were treated with [γ-³²P]-8-azido-ATP and with [γ-³²P]-ATP without pho-

tolysis. Under ATP-hydrolyzing conditions in the presence of 5 mM $MgCl_2$ a number of labeled proteins were identified by autoradiography, but most of these were labeled both in the presence and in the absence of photolysis, indicating that they were phosphorylated proteins and were not specific photolabeled components. This result was not unexpected, since an ATPase might liberate ³²P_i upon ATP hydrolysis and since it has been the experience of others that it is difficult to label ATPases with this analogue under ATP-hydrolyzing conditions.

To obtain specific photolabeling of the nucleotide-binding components, we therefore examined conditions that might permit the binding of ATP but inhibit its hydrolysis to ADP and ³²P_i. Since the pelletable fraction of ATPase could be totally inhibited by adding EDTA in excess of the magnesium concentration, we repeated the labeling procedure in 30 mM Tris-HCl, pH 8.0, containing [γ-³²P]-8-azido-ATP and 10 mM EDTA. In the presence of EDTA the phosphorylation of proteins was nearly eliminated and only a few specific photolabeled proteins were observed (Fig. 1). These included an electrophoretic component similar to actin, and two polypeptides of 50,000 and 87,000 mol wt. A considerable amount of labeled material was also observed in the stacking gel with or without photolysis, but the identity of these materials has not been determined. Since actin ATPase was determined to be negligible in these preparations (see below) and since the labeling of the 50-kdalton component was generally 5–10 times greater than that of the 87-kdalton polypeptide, these observations suggested that the principal ATPase activity might be associated with the 50-kdalton polypeptide.

Release of ATPase Activity from Membranes by Treatment with Dichloromethane

We therefore used Apps's modification (2) of Beechey's procedure of isolating F-1 ATPase from chromaffin granule membranes in which membrane fractions are exposed to chloroform or dichloromethane to release the ATPase into an aqueous phase in a form that is readily soluble. When the pelletable fraction of ATPase from microtubules was exposed to dichloromethane, we observed that substantial amounts of ATPase activity were released. The enzyme was soluble as judged by sedimentation and gel filtration chromatography and was stable for days at 22°C. However, like the F-1 ATPase extracted from chromaffin granules, the ATPase prepared from microtubules was labile at low temperature with an observed half-life of 5 min at 5°C. Analysis of this fraction on SDS gels revealed prominent bands at 50,000 and 43,000 mol wt plus numerous other components (Fig. 2 C).

Although the specific activity of solvent-released enzyme was increased (sp act, 0.4–0.8), the yield of ATPase activity was low and variable, ranging from 10 to 40% of the original total units of activity. Investigation of the residual precipitated protein revealed that not all of the ATPase activity was released by a single extraction and that the yield of activity could be increased by repeated extraction of the protein precipitate. Because of the low efficiency of extraction, it has not been possible to calculate directly how much of the total ATPase activity the solvent-extractible form represents. On the basis of several indirect calculations, however, we estimate that it represents 80–90% of the total ATPase activity (see Discussion).

Identification of a 50-kdalton ATPase by Native Gel Electrophoresis

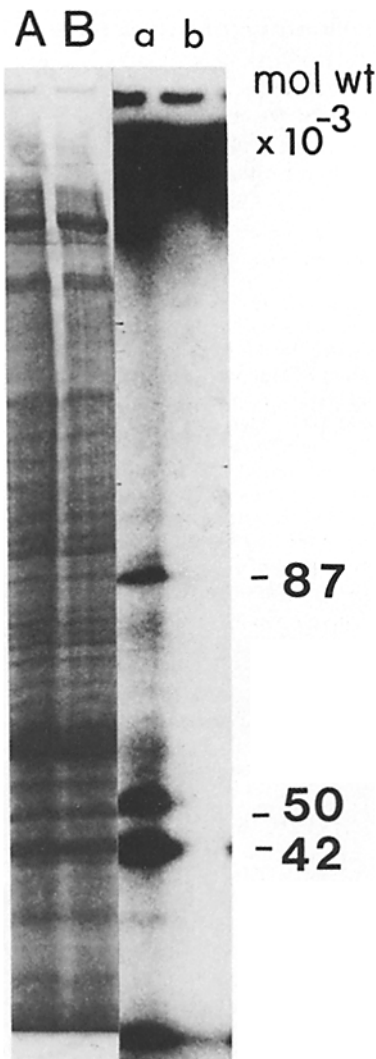To identify the ATPase species in this complex fraction of

A B a b

mol wt ×10⁻³

– 87

– 50
– 42

FIGURE 1 Photoaffinity labeling of the pelletable ATPase with [γ-³²P]-8-azido-ATP. Samples of pelletable ATPase were exposed to [γ³²P]-8-azido-ATP with (A) and without (B) exposure to UV light. A 100-μl volume containing 4 mg/ml pelletable ATPase fraction and 20 μM [γ³²P]-8-azido-ATP (1 × 10⁶ cpm) was mixed for 10 s at 4°C on a white porcelain spot plate and photolyzed for 1 min with a UVS-11 mineral light (Ultra-Violet Products, Inc., San Gabriel, CA) positioned 10 cm from the plate. The samples were fractionated by SDS PAGE and stained with Coomassie Blue to indicate proteins (A and B). Autoradiograms of the same gel were prepared to identify ATP-binding polypeptides (a and b) specifically labeled by photolysis in the presence of photoaffinity analogue.

proteins, we used a combination of native gel and SDS gel electrophoretic procedures. When dichloromethane extract was fractionated on native tube gels and stained for ATPase activity with ATP and the nitrate salts of magnesium and lead, a single intense band of lead phosphate was observed. Parallel gels
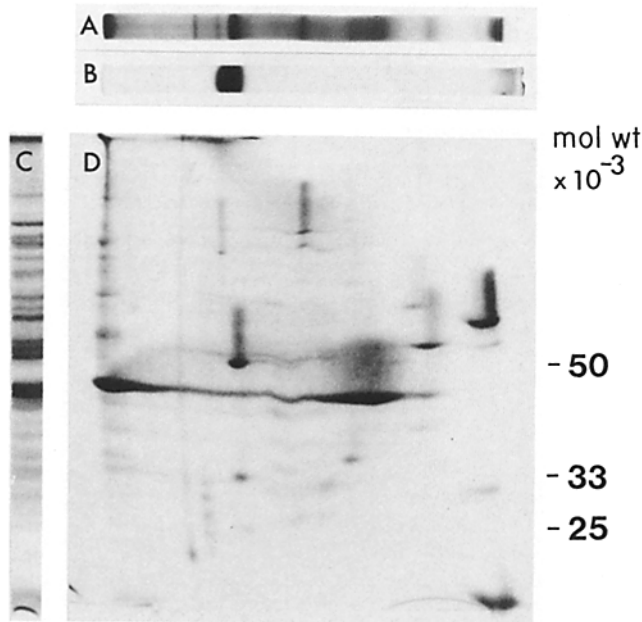
FIGURE 2 Identification of dichloromethane-released ATPase by two-dimensional gel electrophoresis. A sample of dichloromethane-released ATPase was fractionated by native gel and SDS gel electrophoretic procedures and was stained for protein and ATPase activity as described in Materials and Methods. (A and B) Native gels stained for protein (A) and ATPase activity (B). The two gels have been mounted with the tops of the gels to the left. The lead reaction product seen near the dye front (right) is derived from a precipitate of lead chloride and was not seen if Tris-NO₃ was used in place of Tris-Cl during electrophoresis. (C and D) SDS gels mounted vertically. (C) SDS gel of ATPase sample. (D) Two-dimensional gel prepared by electrophoresing proteins from a native tube gel into an SDS slab gel (see Materials and Methods). The single band of ATPase activity on the native gel in (B) is resolved into a 50,000-dalton polypeptide plus smaller amounts of 33,000 and 25,000 mol wt components on the SDS slab gel (D).

stained for protein with Coomassie Blue revealed a major band in the same position on the gel (Fig. 2 A and B). When native gels were diced into 5-mm segments after electrophoresis and assayed for ATPase activity in our standard ATPase assay mixture, it could be shown that the segment containing ATPase activity was in the same position as the band revealed by histochemical and Coomassie Blue staining. In control experiments we also determined that ATPase activity was nearly fully active in the lead nitrate-containing buffer used to indicate ATPase activity on the native gel. Thus, the precipitated band of lead phosphate indicated the site of ATPase activity and was not due to phosphatase activity or to the precipitation of phosphate that had been released from phosphorylated proteins.

The proteins composing the ATPase band on the native gel were identified by electrophoresing the proteins from the native gel into an SDS slab gel (see Fig. 5 D). A major protein with a molecular weight of 50,000 daltons and minor 33-kdalton and 25-kdalton components occupied a position exactly coincident with the band of ATPase activity. Thus, the native gel procedure indicated that the dichloromethane extract contained only one species of ATPase and that this activity was associated with a 50,000-dalton protein.

Purification of a 50-kdalton Protein with ATPase Activity

We purified the ATPase from dichloromethane extract by sedimentation on glycerol gradients using the method of Apps (2). When fractionated by sedimentation on a 8–35% linear glycerol gradient, the ATPase activity was distributed in a single, well-defined peak that migrated in advance of the bulk of other soluble proteins. As shown in Fig. 3, this peak was greatly enriched in the 50-kdalton protein but also contained other components of which the chief contaminant was actin. Much of the actin in the sample pelleted as F-actin and was identified by electron microscopy and by its ability to bind myosin subfragment-1.

The pooled ATPase fraction was further purified by gel filtration chromatography on a column containing 40 ml of 4% agarose pre-equilibrated with TED containing 0.1% Triton. The purified ATPase fraction (sp act 8.6, representing a 716-fold purification) contained a 50-kdalton protein (40%), a 33-
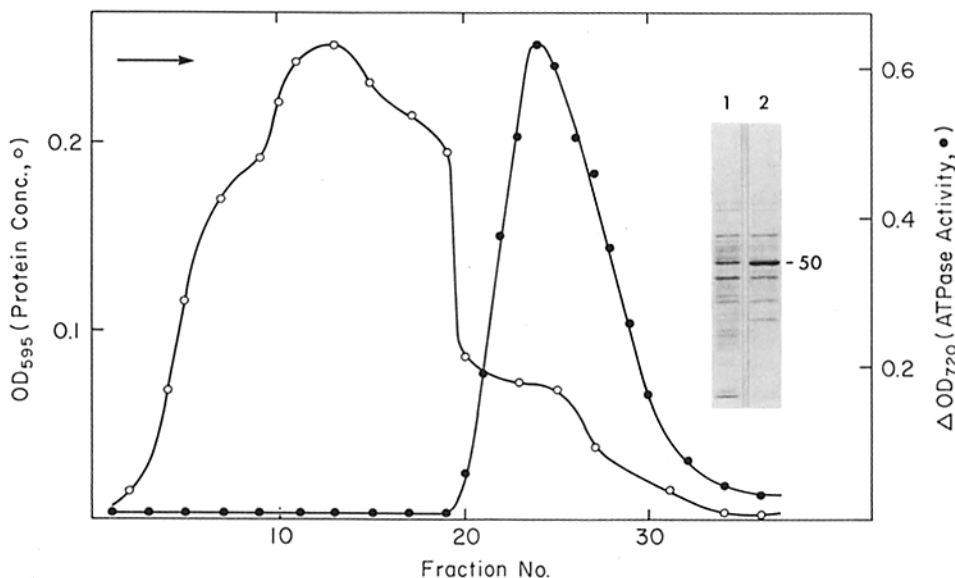
FIGURE 3 Fractionation of dichloromethane-released ATPase by sedimentation on a glycerol gradient. A sample of dichloromethane-released ATPase was layered on an 8–35% linear glycerol gradient in TED and sedimented in a Beckman SW 27 rotor at 100,000 g for 20 h at 25°C. A single well-defined peak of ATPase activity was obtained. SDS gels in inset show sample (1) and resulting ATPase peak (2). The 50-kdalton ATPase was substantially enriched by this procedure.

TABLE I

Purification of 50-kdalton ATPase from Neuronal Microtubules*

Step in purification	Protein		Specific activity	Purification	Units	Yield
	mg	%	U/mg		μmol P_i · min^{-1}	%
Purified microtubules	1,200	100	0.012	1	14.40	100
Pellet fraction	109.1	9.09	0.113	9.3	12.32	85.4
Washed pellet	58.83	4.90	0.218	18.2	12.82	89.0
Solvent extract	4.217	0.35	0.43	35.8	1.813	12.6
Glycerol gradient	0.208	0.017	3.86	321	0.803	5.6
Agarose column	0.040	0.003	8.6	716	0.344	2.4
Pure 50-kdalton ATPase (est.)	—	—	(20)	(1,666)	—	—

* The data are from a particular isolation but are representative of the yield of ATPase from 1.2 g of purified microtubule protein (H₂P). The specific activity of the 50-kdalton polypeptide was estimated from the specific activity (8.6) and percent composition (40%) of the peak ATPase fractions from the agarose column.
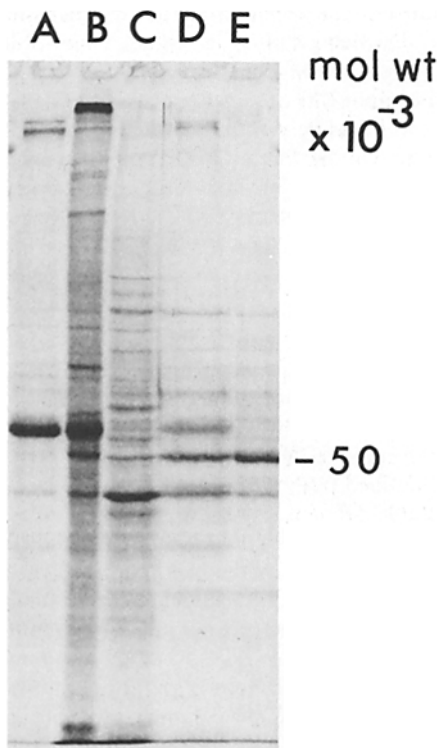

FIGURE 4   Summary of purification of 50-kdalton ATPase. SDS polyacrylamide slab gel demonstrates the composition of fractions at various stages of ATPase purification. (A) Purified microtubule protein with ATPase activity. (B) Pelletable fraction of ATPase. (C) Dichloromethane-released ATPase. (D) Peak ATPase fraction from glycerol gradient. (E) Peak ATPase fraction from agarose column.

TABLE II

Sedimentation of ATPase Activity with Beads of Protein
A-Sepharose Adsorbed with 50-kdalton-specific Antibody

Sample	ATPase activity		
	50-kdalton-specific antibody	IgG flow-through fraction	Preimmune IgG
Supernate	67	3,858	4,120
Pellet	4,450	157	134
Total	4,517	4,015	4,254

Beads of protein A-Sepharose were adsorbed with IgG fractions, and mixed with dichloromethane-released ATPase. After 5 h the Sepharose was sedimented, and the supernate and rinsed Sepharose pellet were assayed for ATPase activity. The volume of mixtures and resuspended pellets was 1.4 ml. ATPase activity is given as cpm/100-μl sample. In this assay 1 U of ATPase activity corresponds to 1.4 × 10⁴ cpm. The 50-kdalton-specific antibody and flow-through fraction of IgG were prepared from a fraction of immune IgG on a 50-kdalton protein-Sepharose affinity column. Preimmune IgG was prepared from serum by cycles of ammonium sulfate precipitation. For details of procedures see Materials and Methods.

kdalton protein (10%) and trace amounts of other proteins. A summary of this purification procedure is shown in Fig. 4 and Table I. From the specific activity of 8.6 and a value of 40% for the percent 50-kdalton protein contained in this fraction by densitometry of SDS gels, we estimated the specific activity of purified 50-kdalton ATPase to be ~20 μmol P_i·mg^{-1}·min^{-1}.

Antibody to the 50-kdalton Subunit Binds to Native ATPase Activity

To confirm that the 50-kdalton polypeptide was associated with ATPase activity, we made an affinity-purified rabbit antibody to the 50-kdalton subunit and examined its ability to bind to native ATPase activity in vitro. Aliquots of 50-kdalton specific and nonspecific antibody fractions were mixed with beads of protein A-Sepharose and the resulting complexes were

examined for their ability to bind ATPase activity by means of an in-vitro-pelleting assay (see Table II).

For control antibody preparations we used preimmune IgG and the flow-through fraction of IgG from the affinity column used to purify 50-kdalton-specific antibody. Sepharose-protein complexes were mixed with dichloromethane extract (sp act 0.5) for various periods of time, pelleted by brief centrifugation, and the resultant supernates and washed Sepharose pellets were examined for ATPase activity (see Materials and Methods for details). In control experiments we demonstrated that the ATPase activity was stable in these buffer conditions for up to 24 h and that the rate of ATP hydrolysis was linear during the course of the assay (50 min).

After 5-h incubation, 99% of the ATPase activity was depleted from the supernate in the tube containing 50-kdalton-specific antibody but none was depleted from supernates containing the control antibody preparations (Table II). Conversely, analysis of the washed Sepharose-protein complexes showed that the preparation with 50-kdalton-specific antibody contained 99% of the initial ATPase activity, and that the control preparations contained essentially no bound ATPase activity. Similar results were obtained when different preparations of purified antibody and ATPase extract were used. Thus, ATPase activity only bound to and pelleted with beads of protein A-Sepharose that were adsorbed with 50-kdalton-specific antibody. These observations support our hypothesis that the 50,000-dalton protein is associated with ATPase activity.

Antibody to the 50-kdalton Polypeptide Cross-reacts with F-1 ATPase from Mitochondria

As shown in Fig. 5 the affinity-purified antibody specifically labeled the 50-kdalton protein in the sample of electrophoretically purified 50-kdalton antigen (B) and in fractions containing a mixture of proteins such as brain extract (E) and the solvent-extracted ATPase (C). The antibody also bound to 50-kdalton polypeptides in a preparation of purified mitochondrial F-1 ATPase (A, F, G, and H), indicating a similarity in the antigenic sites of the 50-kdalton proteins that compose F-1 ATPase and the membrane-associated ATPase from microtubules. With shorter exposure times it could be seen that both the α and β subunits of F-1 ATPase were labeled (not shown).

Comparison of Brain 50-kdalton ATPase from Microtubules with Mitochondrial F-1 ATPase

The observation of cross-reactivity between the 50-kdalton ATPase antibody and the 50-kdalton subunits of mitochondrial ATPase suggested that these enzymes may be related. We therefore compared these ATPases as follows.

SUBUNIT COMPOSITION: The molecular weights of the α, β, and γ subunits of F-1 were determined from Laemmli slab gels to be 50, 49, and 32 kdaltons, respectively, and are in agreement with previously published values (17). In contrast, ATPase from microtubules contained polypeptides of 50 and 33-kdaltons plus a small amount of contaminating actin and other trace components. Although similar, the 50-kdalton ATPase from brain contained only a single 50-kdalton polypeptide and had a 33-kdalton polypeptide that was electrophoretically distinct from the γ-subunit of F-1 ATPase. The significance of these differences has not been investigated.

SOLVENT EXTRACTIBILITY: The 50-kdalton ATPase and F-1 ATPase share the unusual property that they can be released into a soluble form after exposure to solvents such as chloroform or dichloromethane. Both enzyme preparations are stable at 22°C for several days but decay with a half-life of 5 min at 5°C. Similar cold lability of solvent-released F-1 ATPase was reported previously by Apps (2).

SEDIMENTATION COEFFICIENT: The sedimentation coefficient of the 50-kdalton ATPase in dichloromethane extracts was estimated to be 17S by the method of Martin and Ames (20). This value is similar to the values of 12–14S that have been reported for F-1 ATPase from mitochondria (5, 9).

SENSITIVITY TO INHIBITORS OF ATPASE ACTIVITY: Mitochondrial ATPase displays characteristic sensitivities to various inhibitors depending on whether the ATPase is membrane-bound as an F_0-F_1 complex (oligomycin-sensitive form) or exists as dissociated F_1 (sodium azide-sensitive form) (16, 33). We compared the sensitivity of the brain 50-kdalton ATPase and mitochondrial ATPase to these inhibitors in both the bound and free states. For membrane-associated enzyme we used intact mitochondrial membranes and a fraction of pelletable, membrane-associated ATPase from microtubules; for dissociated enzyme we used the dichloromethane extracts of these fractions. Beechey (5) showed previously that the dichloromethane extract of mitochondria has the properties of dissociated F-1 ATPase and is sensitive to sodium azide.

As shown in Table III, the dichloromethane-released ATPase fractions were also nearly completely inhibited by 1 mM azide. However, the membrane-associated ATPase fractions showed differences in sensitivity to oligomycin. In the presence of 10 μM oligomycin, 96% of the mitochondrial ATPase was inhibited as compared to only 25% inhibition for the 50-kdalton ATPase. The significance of the differences in oligomycin sensitivity is discussed below.

Electron Microscopy of Purified 50-kdalton ATPase

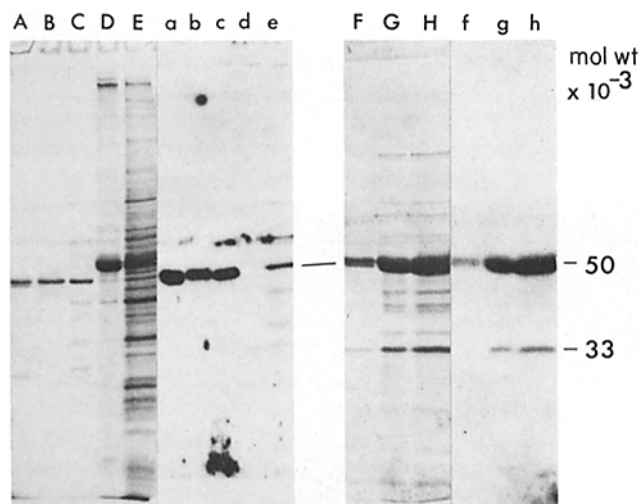The large sedimentation coefficient (17S) suggested that the

FIGURE 5 Examination of the specificity of affinity-purified 50-kdalton antibody by immunoblotting. ATPase fractions were subjected to SDS gel electrophoresis, electrophoretically transferred onto nitrocellulose sheets, and incubated with affinity-purified antibody and ^{125}I-protein A as described in Materials and Methods. SDS gels ($A-E$) and nitrocellulose blots ($F-H$) were stained with Coomassie Blue or amido black, respectively, to indicate proteins. Autoradiograms of nitrocellulose sheets treated with antibody and protein A were prepared to identify crossreactive polypeptides ($a-e$ and $f-h$). A, 0.5 μg mitochondrial F-1 ATPase; B, 0.5 μg 50-kdalton antigen used for the innoculation of rabbits; C, 1 μg partially purified ATPase prepared by extraction with dichloromethane and sedimentation on a glycerol gradient; D, 5 μg microtubule protein containing ATPase activity prepared by two cycles of assembly-disassembly; E, 20 μg extract prepared from brain tissue homogenate (the low level of reactivity to other extract proteins was variable in extent and is thought to be due to the age and activity of the ^{125}I-labeled protein A used to label the blots); $F-H$, 1, 5, and 10 μg F-1 ATPase from rat liver mitochondria. The reactivity observed to the 33-kdalton γ-subunit of F-1 ATPase was consistently observed, but it is not known if it is specific. The sample of microtubule protein contained a faint band at 50,000 mol wt which is not seen in this exposure. Loadings of 3 mg would be required to obtain a band of similar intensity for this exposure time (30 min).

TABLE III

Inhibition of Mitochondrial ATPase and Membrane-associated ATPase from Microtubules by Oligomycin and Sodium Azide

	% Inhibition	
Sample	Sodium azide (10 μM)	Oligomycin (1 mM)
Mitochondrial ATPase	95	96
ATPase from microtubules	99	25

ATPase activity was measured with and without inhibitors of mitochondrial ATPase, 10 μM oligomycin, or 1.0 mM sodium azide. For oligomycin, intact mitochondrial membranes and the pelletable ATPase from microtubules were used. For sodium azide, the respective dichloromethane-released ATPase fractions were used. Inhibition is indicated as the percent of activity observed in the presence of inhibitor compared to the activity in untreated samples.

50-kdalton ATPase occurred in the form of an oligomer. Since the subunits of mitochondrial F-1 ATPase are associated together in the form of distinctive 10-nm diameter hexamers or rosettes (8), we examined the morphology of the brain 50-kdalton ATPase by electron microscopy. When the peak ATPase fractions from glycerol gradients were negatively stained and examined by electron microscopy we observed particles 10 nm in diameter consisting of 3-nm subunits grouped together in the form of a rosette (Fig. 6). Under conditions of optimal orientation and negative staining each rosette appeared to consist of a ring of six subunits grouped around a central space that sometimes appeared as an empty lumen and sometimes appeared to contain an additional subunit.

Localization of 50-kdalton Protein in the PtK-2 Cells by Immunofluorescence Microscopy

We examined the distribution of the 50-kdalton ATPase in PtK-2 cells that were fixed and processed for immunofluorescence microscopy by an indirect staining procedure (see Materials and Methods). In control experiments we determined that the distribution and morphology of mitochondria and membranous organelles in living cells were preserved following fixation in 4% formaldehyde (Fig. 7A) and that fluorescein-labeled goat anti-rabbit antibody (FGAR) preabsorbed on PtK-2 cell monolayers did not stain cellular components (Fig. 7B). Following incubation with 50-kdalton-specific antibody and FGAR, however, we observed bright specific staining of elongate organelles that were determined to be mitochondria

by phase-contrast microscopy (Fig. 7C and D). There was no indication that other components such as small membrane vesicles were stained by this procedure. The staining of mitochondria appeared to be specific, since preincubation of the antibody with a 1–10-fold excess of dichloromethane-released ATPase and incubation of cells with the antibody-protein mixture blocked the staining of mitochondria completely (Fig. 7E and F).

DISCUSSION

Identification of a 50-kdalton Polypeptide with ATPase Activity

We identified the principal ATPase activity with a 50-kdalton protein by four methods: (a) photo-affinity labeling with [γ-^{32}P]-8-azido-ATP, (b) purification following release of ATPase activity by treatment with dichloromethane, (c) native gel electrophoresis and two-dimensional gel analysis of solvent-released ATPase (d) demonstration of specific binding to affinity-purified antibodies to the 50-kdalton polypeptide. Since photoaffinity labeling with [γ-^{32}P]-8-azido-ATP indicated the 50-kdalton component to be an ATP-binding protein, it is possible that the 50-kdalton protein is itself the ATPase and contains the catalytic site for ATP hydrolysis.

Estimation of the Relative Contribution of 50-kdalton ATPase to Total ATPase Activity

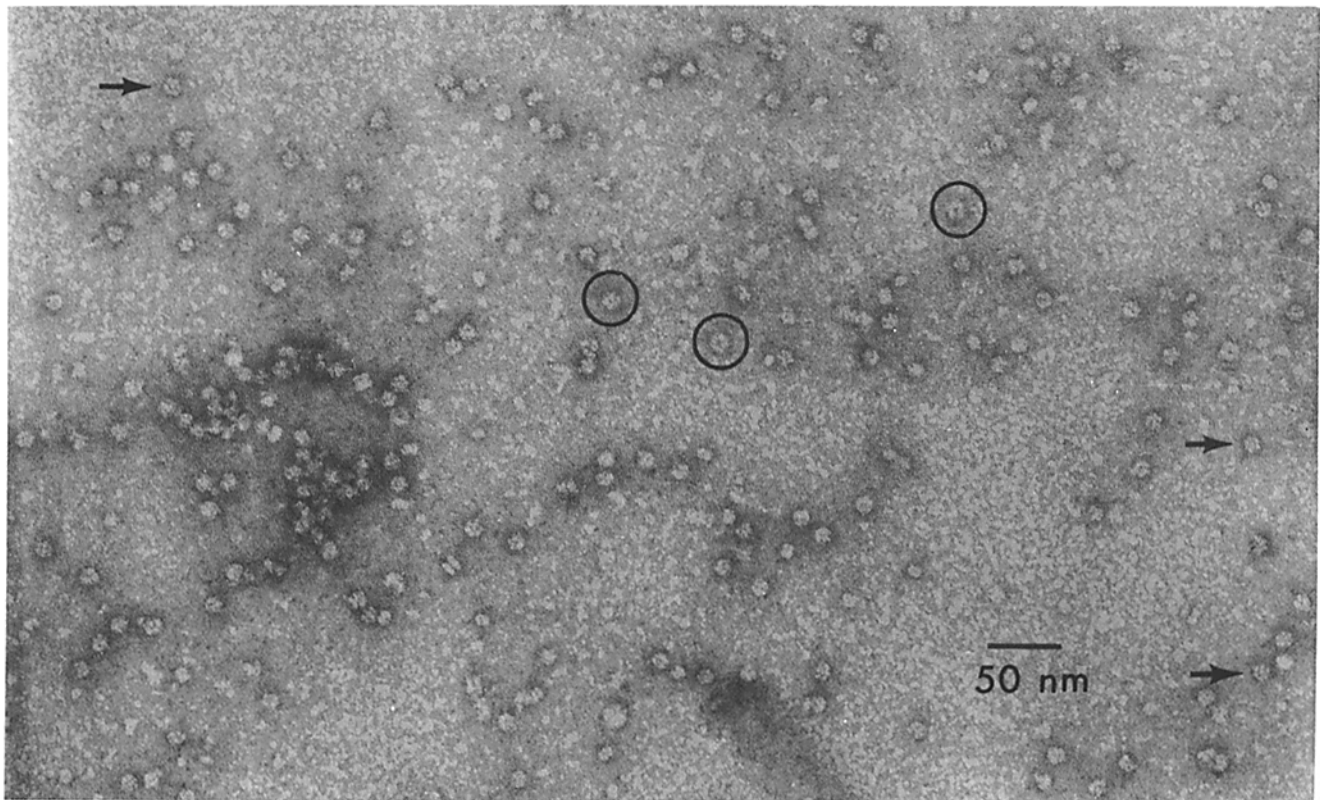It has not been possible to calculate directly how much of

FIGURE 6 Morphology of partially purified ATPase. Dichloromethane-released ATPase, purified by sedimentation on a glycerol gradient, was diluted to 10 μg/ml in TED and negatively stained with 1% uranyl acetate. The fraction contains 10-nm diameter particles each of which is resolved as clusters of six subunits, 2–3 nm in diameter. In favorable views (arrows) six subunits appear to be hexagonally packed around a seventh central component. In other instances a central subunit appears to be missing and the subunits form a ring around an empty lumen (circles). The morphology of these particles is similar to that of isolated F-1 ATPase from mitochondria. Bar, 50 nm. × 200,000.
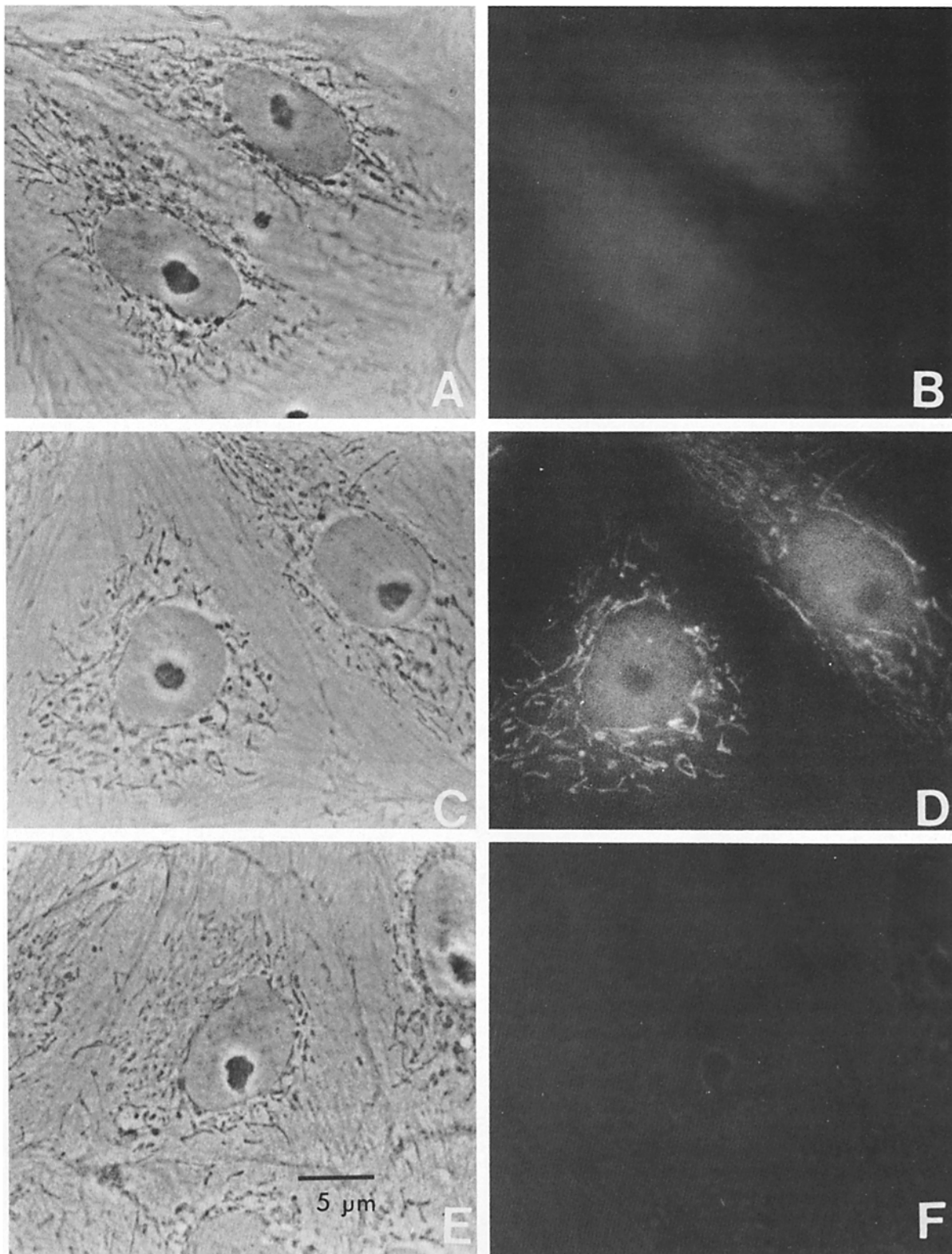
FIGURE 7 Localization of affinity-purified 50-kdalton antibody in PtK-2 cells by immunofluorescence microscopy. Phase and fluorescence micrographs are shown for cells fixed in PBS containing 1 mM MgCl₂ and 4% formaldehyde and processed for indirect immunofluorescence microscopy with affinity purified 50-kdalton antibody and FITC-labeled goat antirabbit antibody (FGAR) as described by Osborn and Weber (23). A and B, cells treated with FGAR alone. C and D, cells treated with 50-kdalton-antibody followed by FGAR. E and F, cells treated with 50-kdalton-antibody preabsorbed with dichloromethane-released ATPase followed by FGAR. For preabsorption, antibody was incubated with an equal amount of ATPase for 120 min and the antibody-protein mixture was used for cell staining. The concentrations of antibody and FGAR are the same for each of the conditions shown. Bar, 5 μm. × 2,500.

the total ATPase activity is due to the 50-kdalton ATPase because of the variability and inefficiency of the dichloromethane extraction procedure. As described above, not all of the ATPase activity is released by a single extraction, since the yield of enzyme can be increased by repeated extraction of the precipitated protein. It is also not known to what extent ATPase activity is changed by bringing the protein from a hydrophobic into an aqueous environment. We therefore used indirect methods for estimating the relative contribution of the 50-kdalton ATPase:

(a) Using intact rat liver mitochondria we recovered 25% of the mitochondrial enzyme activity by extraction with dichloromethane. Assuming a similar efficiency of extraction of the brain 50-kdalton ATPase from the pelletable membrane fraction from microtubules, we estimate that up to 80% of the ATPase activity is due to the 50-kdalton protein.

(b) As reported in the preceding paper (21), up to 90–95% of the brain microtubule ATPase is pelletable and is thought to be associated with membrane vesicles. Virtually all of this activity can be solubilized and recovered by octylglucoside extraction, and 52% of this extracted activity can be released by dichloromethane treatment and shown to be due to the 50-kdalton ATPase.

(c) By densitometry of SDS polyacrylamide gels we determined that 13.8% of the Coomassie-staining material in the pelletable fraction of ATPase is due to the 50-kdalton polypeptide. Assuming a specific activity of 20 for the purified 50-kdalton ATPase, we estimated the specific activity of the pelletable ATPase fraction to be 0.28, which was close to the actual observed value of 0.22.

(d) The values of the estimated proportion of 50-kdalton ATPase (80–90%) are in agreement with the estimated value for the sum of other soluble and membrane-associated ATPases and coupled kinase-phosphatase activities (15–20%). Pseudo-ATPase activity due to coupled kinase-phosphate activities and ATPase activity due to myosin, actin, dynein, and tubulin was found to be negligible. Of the other possible membrane-associated activities, only the Na^+/K^+-ATPase was determined to be significant, contributing 5–10% of the total ATPase activity.

In the past there has been some concern expressed over the possible contribution of an apparent ATPase activity due to the coupled activity of protein kinase and phosphoprotein phosphatase. Although not reported in these papers, we have examined this possibility extensively using $[\gamma-^{32}P]$-ATP in pulse-chase experiments. We observed that although proteins were phosphorylated the turnover of phosphate was negligible even after 1 h, from which we estimated that the contribution of this coupled activity to total ATPase activity was <0.1%. This finding is in agreement with the detailed results presented by White et al. (36).

The Relationship of the 50-kdalton ATPase to F-1 ATPase from Mitochondria

Antibody to the 50-kdalton protein was observed to cross-react strongly with the 50-kdalton polypeptide chains of mitochondrial ATPase. To evaluate the identity or uniqueness of these enzymes we compared the composition and some of the biochemical properties of these ATPases.

(a) The dichloromethane-extracted forms of these ATPases are similar with respect to the presence of major 50,000-dalton polypeptides that contain ATPase activity and share common antigenic sites. However, whereas F-1 ATPase contains two distinct α- and β-chains, with a molecular weight of ~50,000

daltons (9, 17), the 50-kdalton ATPase contains only a single chain with an electrophoretic mobility indistinguishable from the α-chain of F-1 ATPase. The lower molecular weight polypeptides associated with these enzymes also appear to be different. Thus, the two ATPase species appeared to be composed of similar but not identical subunits.

(b) Both the 50-kdalton ATPase and F-1 ATPase have similar physical properties including large sedimentation coefficients and a structure consisting of six subunits arranged in the form of a rosette around a central lumen or protein component. The diameter of the hexamer (10 nm) is the same as that reported previously for F-1 ATPase (8).

(c) The dichloromethane-released forms of both enzymes were determined to have similar biochemical properties, including marked lability at 5°C and sensitivity to sodium azide. However, significant differences were observed in the sensitivities of nonextracted enzyme preparations to oligomycin, a well characterized inhibitor of mitochondrial ATPase in the membrane-associated F_0-F_1 configuration (16). At concentrations of oligomycin that totally inhibit membrane-bound F-1 ATPase (10 μM), the pelletable fraction of ATPase from microtubules was only moderately inhibited. The inhibition we observed varied from preparation to preparation, ranging from 5 to 25%.

Thus, although the 50-kdalton ATPase and F-1 ATPase appear to be similar enzymes, the differences in polypeptide composition and in sensitivity to oligomycin may be significant. It is possible that the 50-kdalton ATPase is in fact mitochondrial in origin and that the procedures used for microtubule purification and storage have altered some of its properties. However, an alternate idea is that the 50-kdalton ATPase is related to but distinct from mitochondrial F-1 ATPase and that is is derived from membrane vesicles other than mitochondria. There is precedent for such an interpretation in the studies of Apps and collaborators (2, 3, 4) who partially purified an F-1-like ATPase activity from chromaffin granule membranes. Although the chromaffin granule ATPase was similar to mitochondrial ATPase, it could be distinguished from mitochondrial F-1 ATPase by its lack of sensitivity to oligomycin and reduced sensitivity to other antibiotics. Toll and Howard (30) have also reported that ATPase and proton motive force may function in the transport of acetylcholine into storage vesicles in brain tissue. Thus, it is possible that the 50-kdalton ATPase in our microtubule preparations may be derived from various membrane sources, including mitochondria, synaptic vesicles, and amine storage granules.

Distribution of 50-kdalton Protein in Intact Cells

In the first paper of this series (21) we reported that most of the ATPase activity in fractions of pelletable ATPase associated with microtubules prepared by in vitro assembly may be contained in membrane vesicles. We have been able to confirm this hypothesis in the present study by showing that mitochondria in PtK-2 cells are specifically labeled when cells are treated with 50-kdalton-antibody and examined by immunofluorescence microscopy. Our observation that mitochondria were specifically stained in these cells strongly suggests that the principal ATPase activity in microtubule preparations is associated with membranes.

Although other membrane components such as 50–100-nm vesicles were not identified as sites of the 50-kdalton protein in PtK-2 cells, our observations do not rule out such a possibility, since the detection of fluorescence by light microscopy is limited by the density of antigen and the resolution afforded

by this technique. Thus, small vesicles containing relatively low amounts of antigen might not be detected by this method. Thus, our studies do not provide an unambiguous answer regarding the uniqueness of the 50-kdalton ATPase from F-1 ATPase or its site of origin in the cell, and further studies would be required to determine whether the 50-kdalton ATPase in microtubules is derived from mitochondria or other membrane components.

The Molecular Basis for Microtubule-dependent Movements Remains Unclear

Our demonstration of the homology of the 50-kdalton ATPase with F-1 ATPase strongly suggests that the ATPase activity in microtubule preparations is associated with membrane vesicles such as small storage granules or mitochondrial fragments. It is possible that this membrane-associated ATPase activity may play a role in vesicle-microtubule interactions, an idea first proposed by Schmitt (26). However, since the 50-kdalton ATPase appears to be closely related to F-1 ATPase, and since the function of F-1 in maintaining hydrogen ion gradients across vesicle membranes is well known, it is difficult to imagine how such an activity could be related to the binding of vesicles to microtubules or their transport and movement within the cells. These studies also do not support our initial hypothesis that microtubule-dependent movements are determined by a specific microtubule-associated ATPase such as dynein. It is possible that microtubule- or membrane-associated ATPases involved in cell motility do not copurify with microtubules during cycles of in vitro purification, in which case we would not expect to detect them. However, to the extent that preparations of microtubule protein prepared by in vitro assembly methods do contain the full complement of proteins required for microtubule function in vivo, these observations suggest that an alternative mechanism may be involved in microtubule-dependent movements.

Note

Since this paper was submitted, Tominaga et al. have reported on two ATPases in preparations of bovine brain microtubules (*FEBS* [*Fed. Eur. Biochem. Soc.*] *Lett.* 144:112–116).

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