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

DOUGLAS B. MURPHY, KATHLEEN T. WALLIS, and RONALD R. HIEBSCH
Department of Cell Biology and Anatomy, Johns Hopkins University, School of Medicine, Baltimore, Maryland 21205

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-32P]-8-azido-ATP; (b) analysis of two-dimensional gels (native gel × 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 Pi·mg⁻¹·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-32P]-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 Pi·mg⁻¹·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-SO4, pH 7.7, containing 1.0 mM EDTA and DTT (TED), sedimented as above to remove remaining soluble proteins, and 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 80-90% of the initial units of ATPase activity present in samples of in-vitro-purified microtubules.

DETERTGEN-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% DTT 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). Detergent-soluble 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-N03, pH 8.0, containing 5 mM Mg(NO3)2, 1 mM Pb(NO3)2, and 3 mM ATP according to the procedure of Wachtstein and Meisel (34). After maximal development of the lead phosphate band (1-2 h), gels were dried and stored in distilled water. For autoradiography, 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 Gailly 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. Antisera 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 125I-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 125I-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 50-kdalton protein-Sepharose Cl4B (Pharmacia, 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 resuspended in 10 ml of 30 mM Tris-HCl, pH 8.0 with 0.1 mM EDTA and 1.2 mM MgCl2 to remove unbound protein, transferred, and centrifuged to a 1.5 ml Eppendorf tube, rinsed with 20 μl of 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 biochemicals 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,
tein kinase activity, we also examined samples that were treated
labeled from those that were simply phosphorylated by pro-
photolysis. To distinguish those proteins that were specifically pho-

fraction were mixed with 20 μM 8-azido-ATP, photolyzed for
one-third of the enzymatic sites in the preparation.

photolabeling in the presence of analogue appeared to block
photolysis had little or no effect on ATPase activity. Thus, 8-
whereas exposure to UV light alone or to 8-azido-ATP without

azido-ATP is utilized as efficiently as ATP by the ATPase and

fraction of microtubule ATPase utilized 110% 8-azido-ATP as
well as ATP, and that the enzyme has a Km of ~12 μM at 0°C
in the buffer used to assay ATPase activity. In addition,

binding subunits of flagellar dynein and mitochondrial F-1

have been described previously by Haley and colleagues (10, 13, 14,
15). A report on the use of this analogue to identify ATP-

Gel Electrophoresis

Preparations of brain extract and the pelletable ATPase

preparation were exposed to
photolytic products, Inc., San

were mixed for 10 s at 4°C on

microtubules was labile at low temperature with an observed

residual precipitated

protein revealed that not all of the ATPase activity was released

units of activity. Investigation of the residual precipitated

by a single extraction and that the yield of activity could be

by SDS PAGE and stained with Coo-

10 cm from the plate.

for 1 rain with a UVS-11

plate and photolyzed

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

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

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 obser-

vations 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 App's modification (2) of Beechey's
procedure of isolating F-1 ATPase from chromaffin granule
membranes in which membrane fractions are exposed to chloro-

roform 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

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 obser-

vations suggested that the principal ATPase activity might be

associated with the 50-kdalton polypeptide.

Identification of a 50-kdalton ATPase by Native
Gel Electrophoresis

To identify the ATPase species in this complex fraction of
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 stained for protein with Coomassie Blue revealed a major band in the same position on the gel (Fig. 2A 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 Materials and Methods). 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-
TABLE I

Purification of 50-kdalton ATPase from Neuronal Microtubules *

| Step in purification       | Protein | Specific activity | Purification | Units | Yield |
|---------------------------|---------|------------------|--------------|-------|-------|
|                           | mg      | %                | U/mg         | µmol P - 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 | 5.6  |
| 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.

TABLE II

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

| ATPase activity | 50-kdalton-specific IgG | IgG flow-through fraction | Preimmune IgG |
|-----------------|--------------------------|---------------------------|---------------|
| Sample          |                          |                           |               |
| 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 mixed 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 x 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.

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.

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 F0-F1 complex (oligomycin-sensitive form) or exists as dissociated F1 (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 ATPases were associated with membranes. However, the relatively large size of these ATPases indicated that they were not likely to be associated with the density of 12S mitochondria. The observation of cross-reactivity between the 50-kdalton ATPase antibody and the 50-kdalton subunits of mitochondrial ATPase suggested the similarity of these enzymes. 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 F0-F1 complex (oligomycin-sensitive form) or exists as dissociated F1 (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.
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-32P]-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-32P]-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%). Pseudophosphorylation of 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 [\(\gamma^3P\)]-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 \(\alpha\)- and \(\beta\)-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 \(\alpha\)-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_{o}F_{1} configuration (16). At concentrations of oligomycin that totally inhibit membrane-bound F-1 ATPase (10 \(\mu\)M), 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 it 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. Low amounts of antigen might not be detected by this method. A simple and rapid method for the preparation of adenosine triphosphate from submicrotubule particles. Biochem. J. 146:553-557.

Bennett, G. V., and P. Stembeck. 1979. Identification and partial purification of asynkin, the high affinity attachment site for human erythrocyte spectrin. J. Biol. Chem. 254:2533-2540.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Catterall, W. A., and P. L. Pedersen. 1974. Structural and catalytic properties of mitochondrial adenosine triphosphatase. Biochem. Soc. Symp. 44:83-88.

Catterall, W. A., and P. L. Pedersen. 1975. Adenosine triphosphatase from rat liver mitochondria. I. Purification, homogeneity and physical properties. J. Biol. Chem. 250:4987-4994.

Crompton, J. R. Gaethlin, and B. Hailey. 1979. Synthesis and use of azide photoaffinity and analogues of adenosine and guanine nucleotides. Methods Enzymol. 56:642-653.

Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry. 10:2656-2671.

Galilay, R., and J. S. Garvey. 1968. Primary stimulation of rahn and mice with hemocyanin in solution and adsorbed on bentonite. J. Immunol. 101:924-929.

Halsey, B. E. 1975. Photoaffinity labeling of adenosine-3',5'-cyclic monophosphate binding sites of human red cell membranes. Biochemistry. 14:3852-3857.

Hailey, B. E., and J. F. Hoffman. 1974. Interactions of a photoaffinity ATP analogue with cation-stimulated adenosine triphosphatases of human red cell membranes. Proc. Natl. Acad. Sci. USA. 71:3367-3371.

Hoyet, P. B., J. R. Owusu, and B. E. Hailey. 1980. The use of photoaffinity probes to elucidate molecular mechanisms of nucleotide-regulated phenomena. Ann. N.Y. Acad. Sci. 346:280-301.

Lowery, F., and E. D. Slyater. 1961. The use of colcemid as an inhibitor of oxidative phosphorylation. J. Biochem. 49:499-501.

Knowles, A. F., and H. S. Penefsky. 1972. The subunit structure of beef heart mitochondrial ATPases. J. Biol. Chem. 247:624-630.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London). 227:680-685.

Lowery, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

Martin, R. G., and B. N. Ames. 1961. A method for determining sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. 236:1372.

Murphy, D. B., R. B. Beechey, S. A. Hubbard, P. E. Linnett, A. D. Mitchell, and E. A. Munn. 1975. A simple and rapid method for the preparation of submicrotubule particles. Biochem. J. 146:553-557.

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).

REFERENCES

1. Anker, H. S. 1970. A solubilizable acrylamide gel for electrophoresis. FEBS (Fed. Eur. Biochem. Soc.) Lett. 7:293.

2. Appes, D. K., and L. A. Glover. 1978. Isolation and characterization of magnesium adenosinetriphosphatase from the chromaffin granule membrane. FEBS (Fed. Eur. Biochem. Soc.) Lett. 55:254-258.

3. Appes, D. K., J. G. Fryde, R. Sutton, and J. H. Phillips. 1980. Inhibition of adenosine triphosphatase, 5-hydroxytryptamine transport and proton-translocation activities of resealed chromaffin-granule "ghost" preparations. Biochem. J. 190:909-917.

4. Appes, D. K., and G. Satcz. 1979. An adenosine triphosphatase isolated from chromaffin granule membranes is closely similar to F-1 adenosine triphosphatase of mitochondria. FEBS Lett. 100:411-419.

5. Beechey, R. B., S. A. Hubbard, P. E. Linnett, A. D. Mitchell, and E. A. Munn. 1975. A simple and rapid method for the preparation of adenosine triphosphatase from submicrotubule particles. Biochem. J. 146:553-557.

6. Bennett, G. V., and P. Stembeck. 1979. Identification and partial purification of asynkin, the high affinity attachment site for human erythrocyte spectrin. J. Biol. Chem. 254:2533-2540.