Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 48–52, January 1986
Biochemistry

Structural studies of the vacuolar membrane ATPase from *Neurospora crassa* and comparison with the tonoplast membrane ATPase from *Zea mays*

(H⁺-translocating ATPase/vacuoles/acidic vesicles)

EMMA JEAN BOWMAN, SUZANNE MANDALA, LINCOLN TAIZ, AND BARRY J. BOWMAN

Thimann Laboratories, Department of Biology, University of California, Santa Cruz, CA 95064

Communicated by Harry Beevers, August 29, 1985

ABSTRACT **The H⁺-translocating ATPase located on vacuolar membranes of *Neurospora crassa* was partially purified by solubilization in two detergents, Triton X-100 and *N*-hexadecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate, followed by centrifugation on sucrose density gradients. Two polypeptides of M_r ≈70,000 and ≈62,000 consistently migrated with activity, along with several minor bands of lower molecular weight. Radioactively labeled inhibitors of ATPase activity, *N*-[^{14}C]ethylmaleimide and 7-chloro-4-nitro[^{14}C]benzo-2-oxa-1,3-diazole, labeled the M_r ≈70,000 polypeptide; this labeling was reduced in the presence of ATP. *N*,*N*′-[^{14}C]dicyclohexylcarbodiimide labeled a polypeptide of M_r ≈15,000. Estimation of the functional size of the vacuolar membrane ATPase by radiation inactivation gave a value of M_r 5.2 × 10⁵, 10–15% larger than the mitochondrial ATPase. The *Neurospora* vacuolar ATPase showed no crossreactivity with antiserum to plasma membrane or mitochondrial ATPase but strongly crossreacted with antiserum against a polypeptide of M_r ≈70,000 associated with the tonoplast ATPase of corn coleoptiles. These results suggest that fungal and plant vacuolar ATPases may be large multisubunit complexes, somewhat similar to, but immunologically distinct from, known F_0F_1 ATPases.**

The vacuoles of *Neurospora crassa* are small organelles characterized by a high content of basic amino acids, polyphosphate, and degradative enzymes (1). A H⁺-ATPase located in the vacuolar membrane is believed to acidify the interior of the vacuole and also to provide the energy for the accumulation of amino acids. By using inhibitors, vacuolar ATPase can be distinguished from the plasma membrane and mitochondrial ATPases. The vacuolar enzyme is insensitive to vanadate and azide, diagnostic inhibitors for the plasma membrane and mitochondrial ATPases, respectively, but is inhibited by high concentrations of KNO_3 and KSCN (2). In several characteristics—substrate affinity, substrate specificity, and sensitivity to inhibitors of proton translocation or ATP analogs—the vacuolar ATPase resembles the mitochondrial ATPase. Like the mitochondrial ATPase, the vacuolar enzyme appears to contain a small polypeptide (M_r ≈15,000) that binds *N*,*N*′-dicyclohexylcarbodiimide (DCCD) (2, 3).

ATPases with properties similar to those of the *Neurospora* vacuolar enzyme have been described in vacuolar membranes of plants (4) and the yeast *Saccharomyces cerevisiae* (5) as well as in a number of animal organelles, including secretory granules (6), lysosomes (7), and coated vesicles (8). The yeast vacuolar ATPase was reported recently to contain two major polypeptides of M_r 89,000 and 64,000 and a DCCD-binding polypeptide of M_r 19,500 (9). Similarly, partial purification of the ATPase from tonoplast membranes of corn coleoptiles revealed two major polypeptides of M_r 72,000 and 62,000. The ATPase from corn appeared to be a large oligomeric complex since the functional size of the enzyme, measured by radiation inactivation, was about 400,000 daltons (10). In a preliminary investigation, we also observed that polypeptides of M_r ≈70,000 and ≈62,000 were enriched in fractions with high levels of vacuolar membrane ATPase activity (3).

In this investigation we have examined the structure of the *Neurospora* vacuolar membrane ATPase by partial purification, inhibitor-labeling studies, and radiation inactivation analysis. We have also tested for immunological crossreactivity with antibodies to the mitochondrial and plasma membrane ATPases and to the tonoplast ATPase of corn.

MATERIALS AND METHODS

Growth of Cells and Preparation of Membranes. Mitochondria and vacuoles were prepared from wild-type strain RL21a of *N. crassa* by using the "bead beater" procedure as described (2). Vacuoles were lysed by suspending them in 1 mM EGTA (pH 7.5) containing chymostatin (2 μg/ml), and the suspension was centrifuged 10 min at 10,000 × *g*. The resultant pellet, gray with a pink layer on top, contained residual cell wall material and contaminating mitochondria and was discarded. Vacuolar membranes were collected as a small, bright orange pellet by centrifuging the 10,000 × *g* supernatant at 250,000 × *g* for 35 min in a Beckman Ti50 rotor, suspended, and stored as described (2). From 12 liters of cells we obtained 1–2 mg of vacuolar membrane protein with ATPase specific activities of 2–5 μmol/min per mg of protein; the ATPase activity was inhibited at least 90% by DCCD (0.1 mM) or KSCN (100 mM). By several criteria, mitochondrial contamination was not detectable (ref. 2; also see Fig. 4*B* in *Results*). Plasma membranes were prepared by the snail enzyme procedure (11).

Partial Purification of Membrane ATPases. Vacuolar membranes (≈2 mg of protein) were pelleted by centrifuging 12 min in a Beckman Airfuge. The pellets were suspended to a protein concentration of 3 mg/ml in solubilization buffer containing 15% glycerol, 1 mM EDTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg of chymostatin per ml, and 10 mM Tris base, pH adjusted to 7.5 with HCl. Triton X-100 was added from a 10% stock solution to give a concentration of 0.4% (wt/vol). After centrifugation for 15 min in the Airfuge, the 0.4% Triton X-100 supernatant was removed and saved. The pellet was suspended in solubilization buffer (one-third the original volume) and reextracted by adding 10% *N*-hexadecyl-*N*,*N*-dimethyl-3-ammonio-1-pro-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: DCCD, *N*,*N*′-dicyclohexylcarbodiimide; ZW3-16, *N*-hexadecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; MalNEt, *N*-ethylmaleimide.

panesulfonate (ZW3-16) to a final concentration of 0.4%. Again, the mixture was centrifuged 15 min in the Airfuge, and the 0.4% ZW3-16 supernatant was collected. Each of the detergent supernatants was layered on a 10–30% linear sucrose gradient (4.6-ml volume, prepared in solubilization buffer without glycerol) and centrifuged at 230,000 × *g* for 5.5 hr in a Sorvall AH650 rotor.

The F_0F_1 ATPase from *Neurospora* mitochondria was partially purified, essentially by the procedure of Rott and Nelson (12). Tonoplast membranes and partially purified tonoplast ATPase were prepared from corn (*Zea* mays) coleoptiles as described (10).

Labeling of Polypeptides with Radioactive Inhibitors. An 80-μl volume of membranes (50–80 μg of protein) or partially purified ATPase (5–10 μg of protein) was incubated 90 min in an ice bath with [^{14}C]DCCD (0.38 μCi; 1 Ci = 37 GBq; 100 μM DCCD). The mixture was then subjected to gel electrophoresis and fluorography to detect which polypeptides bound the radioactive label. Labeling efficiency and specificity were comparable to results obtained when the incubation volume was 10-fold greater and membranes were separated from unbound radioactive DCCD by centrifugation prior to electrophoresis (2). This procedure was also used to label membranes with 7-chloro-4-nitro[^{14}C]benzo-2-oxa-1,3-diazole ([^{14}C]NBD-Cl) (0.1–0.25 μCi; 25–50 μM NBD-Cl; 60-min incubation). Before labeling membranes with *N*-[^{14}C]ethylmaleimide ([^{14}C]MalNEt) (0.2 μCi; 0.05–0.1 mM MalNEt; 30-min incubation), membranes were preincubated with 0.25 mM unlabeled MalNEt in the presence of 5 mM MgADP (to saturate nonspecific MalNEt-binding sites; ref. 13).

Antisera. Antiserum to the M_r 72,000 and 62,000 polypeptides associated with the ATPase in corn coleoptile tonoplasts (10) was prepared by the procedure of Vaitukaitis *et al.* (14) with some modifications. Approximately 900 μg of deoxycholate-washed tonoplasts was electrophoresed on a $NaDodSO_4$/polyacrylamide gel (8%). After staining the gel with Coomassie blue, the bands were cut out and thoroughly homogenized in 1 ml of 0.15 M NaCl in a glass homogenizer. The homogenates were spun on a Vortex 5–10 min with 1 ml of complete Freund's adjuvant and 5 mg of tubercle bacilli and were further mixed by passage through a 23-gauge syringe needle. This mixture was injected intradermally at about 20 locations along the back of a New Zealand White rabbit. One month later, the rabbit was given six subcutaneous injections of protein prepared as described above. Ten days later blood was collected from the ear vein. Additional injections were given at intervals of ≈2 months, followed by bleedings (8–30 ml collected) 10 days later. (Our attempts, at the same time, to raise antibody to the M_r ≈70,000 polypeptide of the *Neurospora* vacuolar membrane did not succeed.) Antisera were tested for immunological crossreactivity with membrane proteins by the blotting procedure of Rott and Nelson (12).

Antiserum to the plasma membrane ATPase of *N. crassa* was a gift from Carolyn Slayman of Yale University, and antisera to the F_1 portion and the β subunit of the *Escherichia coli* F_0F_1 ATPase were donated by Robert Simoni of Stanford University.

Radiation Inactivation. To compare the functional sizes of the ATPases, *N. crassa* mitochondria and vacuolar membranes were suspended in medium containing 2 mM ATP, chymostatin (2 μg/ml), and 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, adjusted to pH 7.5 with NaOH. The samples were then frozen in dry ice and subjected to irradiation with ^{60}Co as described (10, 15). For irradiation of partially purified ATPases, sucrose density-gradient fractions were used.

Electrophoresis. Our previously described procedure for electrophoresis (16) was used with some modifications. Samples were prepared by adding phenylmethylsulfonyl fluoride (final concentration, 0.5 mM) and $NaDodSO_4$ reagent. After heating at 70°C for 20 min, the samples were electrophoresed on 8%, 10%, or 12% polyacrylamide gels, followed by staining with Coomassie blue or silver. The amounts of M_r ≈70,000 and ≈62,000 polypeptides in vacuolar membranes and gradient fractions were estimated on silver-stained gels with a gel-scanning accessory on a Cary 210 spectrophotometer. Molecular weights of polypeptides in the partially purified vacuolar ATPase were estimated from a standard curve (least-squares fit) of R_f values for "low molecular weight standards" from Bio-Rad.

ATPase Activity Assays and Protein Determination. Vacuolar membrane ATPase activity was assayed as described (2). Protein in membrane fractions was determined according to Lowry *et al.* (17) after suspension in 0.4% sodium deoxycholate. For determinations in sucrose gradient fractions, protein was precipitated with 7% trichloroacetic acid and solubilized in 0.5 M NaOH.

Materials. [^{14}C]DCCD was from Amersham; [^{14}C]NBD-Cl was from Research Products International (Mt. Prospect, IL); and [^{14}C]MalNEt was from New England Nuclear. Reagents for electrophoresis were from Bio-Rad. The zwitterionic detergent ZW3-16 was bought from Calbiochem. Nucleotides, buffers, chymostatin, and Triton X-100 were from Sigma.

RESULTS

Partial Purification of Vacuolar Membrane ATPase. To solubilize ATPase activity, vacuolar membranes of *Neurospora* were subjected to two successive detergent extractions. First, vacuolar membranes (3 mg of protein per ml) were suspended in 0.4% Triton X-100. After centrifugation, 25–35% of the ATPase activity was recovered in the supernatant fraction and 30–50% was recovered in the pellet. (Higher concentrations of Triton X-100 did not increase the amount of activity in the supernatant.) Second, the pellet was suspended in 0.4% ZW3-16, yielding a further recovery of 20–30% of the ATPase activity in a supernatant fraction. Each of the detergent supernatants was then centrifuged on a 10–30% sucrose gradient. ATPase activity formed a symmetrical peak in the lower half of each gradient, whereas ≈60% of the protein remained in the upper half (ZW3-16 gradient shown in Fig. 1 *Upper*; Triton X-100 gradient not shown). Recovery of ATPase activity from the gradients was 60–70% from the 0.4% Triton X-100 supernatant and 25–40% from the 0.4% ZW3-16 supernatant. Protein recoveries were 90–100%.

From analysis of sucrose gradient fractions on $NaDodSO_4$/polyacrylamide gels, two prominent bands, at approximate M_rs of 70,000 and 62,000, were found to copurify consistently with ATPase activity (Fig. 1 *Lower*, bands a and b). The intensities of these bands, stained with either silver or Coomassie blue, were ≈2:1. Several minor bands (M_rs of 52,000, 39,000, 29,000, 27,000, and 16,000) also migrated with ATPase activity on both gradients.

Because of the loss of activity following solubilization, partially purified vacuolar ATPase preparations did not show an increase in specific activity over the starting membranes (2–5 μmol/min per mg of protein). However, by scanning gel lanes and assuming that the M_r ≈70,000 and ≈62,000 polypeptides are components of the enzyme, we estimated the purification to be ≈5-fold. Attempts to increase activity by adding phospholipids—including phosphatidylcholine, phosphatidylserine, and phospholipids extracted from *Neurospora* vacuolar membranes—have thus far been only partially successful, with 30–50% stimulation obtained.

A number of other detergents were tested for use in purifying the vacuolar membrane ATPase. With some deter-

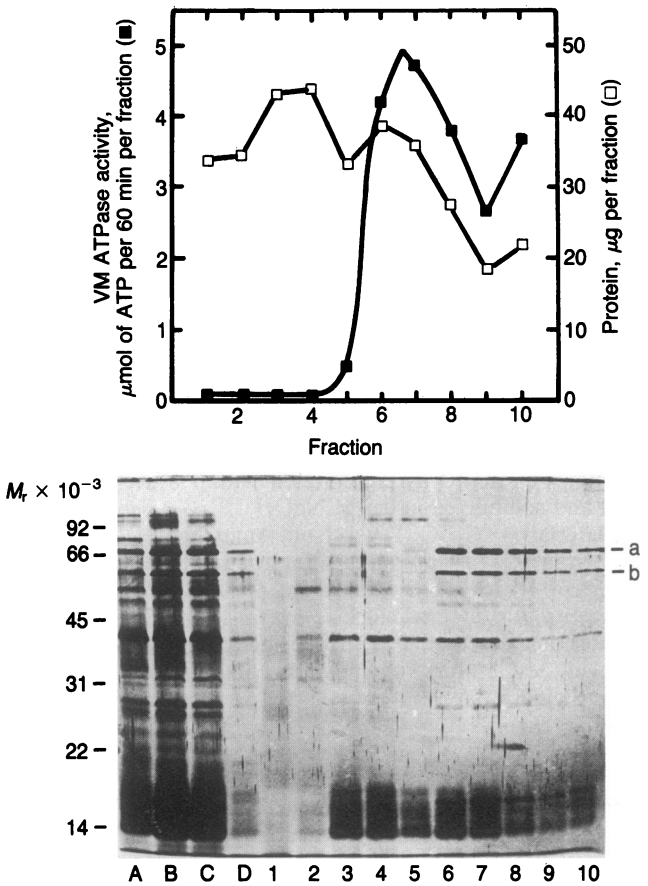

FIG. 1. Partial purification of the vacuolar membrane ATPase. (*Upper*) Distribution of vacuolar membrane (VM) ATPase activity and protein on sucrose gradients loaded with 0.15 ml of ZW3-16 supernatant. (*Lower*) NaDodSO$_4$/polyacrylamide gel (12%) stained with silver. Lanes contained vacuolar membranes (5 μg of protein, lane A), Triton X-100 pellet (8 μg of protein, lane B), ZW3-16 supernatant (7 μg of protein, lane C), ZW3-16 pellet (4 μg of protein, lane D), and 30 μl of gradient fractions 1–10 (lanes 1–10). The calculated M_rs of bands a and b were ≈70,000 and ≈62,000. Values for molecular weight markers are given on the left.

gents {octyl-β-D-glucopyranoside, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, sodium cholate, and deoxycholate}, recovery of activity in the detergent supernatant plus pellet was low, <30%. With others (lysolecithin and ZW3-16 before Triton X-100), recovery of activity in the detergent supernatant was high (60–80%), but little separation of activity from protein occurred on sucrose gradients. Results with octaethylene glycol dodecyl ether and *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate were similar to those with Triton X-100. With all of these detergents, when partial purification was obtained, prominent polypeptides of M_r ≈70,000 and ≈62,000 were always associated with ATPase activity.

Binding of ^{14}C-Labeled Inhibitors. We reported previously that MalNEt and NBD-Cl inhibit activity of the *Neurospora* vacuolar membrane ATPase (2, 3). These inhibitors have been shown by other investigators to bind covalently to specific subunits of other membrane ATPases (13, 18, 19). In preliminary experiments, we found that adenylates could protect against inhibition by MalNEt and NBD-Cl, suggesting that the inhibitors may bind at or near the active site of the vacuolar ATPase. To test for specific binding, vacuolar membranes were incubated with [^{14}C]MalNEt or [^{14}C]NBD-Cl in the presence and absence of 5 mM MgADP (MalNEt) or MgATP (NBD-Cl) and then electrophoresed on NaDodSO$_4$/polyacrylamide gels. Subsequent fluorography showed that both inhibitors preferentially labeled a M_r ≈70,000 polypeptide and that the labeling was partially prevented by adenylates (band "a" in Fig. 2 *A* and *B*). The addition of KSCN, an inhibitor of vacuolar membrane ATPase activity, also prevented the labeling of the M_r ≈ 70,000 polypeptide with [^{14}C]NBD-Cl (Fig. 2*B*, lane 5 vs. lane 1).

Previously we observed that [^{14}C]DCCD, an inhibitor of proton translocation, primarily labels a single polypeptide of M_r ≈15,000 in vacuolar membranes and suggested this protein as a putative component of the vacuolar membrane ATPase (2). Using partially purified ATPase, we found that ATPase activity was completely inhibited by 0.1 mM DCCD and that it also specifically labeled a polypeptide of M_r ≈15,000.

Determination of Functional Size. Radiation inactivation allows a measurement of the functional size of an enzyme without requiring purification of the enzyme. To apply this method to *Neurospora* ATPases, mitochondria and vacuolar membranes were exposed to ^{60}Co, and the loss of ATPase activity was followed as a function of the time of exposure (or dose of irradiation). Molecular weights were calculated by measuring the effect of irradiation on enzymes of known molecular weight, glucose-6-phosphate dehydrogenase (M_r 104,000) and the Ca^{2+}-ATPase of sarcoplasmic reticulum (M_r 230,000) (discussed in ref. 15). As shown in Fig. 3, the Ca^{2+}-ATPase was inactivated at a rate 2.3-fold greater than the inactivation of glucose-6-phosphate dehydrogenase, as would be predicted by their relative molecular weights. Inactivation of the mitochondrial ATPase indicated a M_r of ≈3.9×10^5. Surprisingly, an even larger M_r, ≈4.9×10^5, was measured for the vacuolar ATPase. From a number of determinations, we found an average M_r for the mitochondrial ATPase of 4.6×10^5 (5 experiments) and of 5.2×10^5 for the vacuolar membrane ATPase (10 experiments). In addition, sizes almost identical to their membrane-bound forms were obtained for partially purified mitochondrial and vacuolar ATPases (data not shown).

Immunological Distinctiveness of the Vacuolar ATPase. Although the vacuolar membrane ATPase has properties distinct from other H^+-ATPases of *Neurospora*, the possibility of a close relationship, particularly with the mitochondrial ATPase, remained. We employed electrophoretic trans-

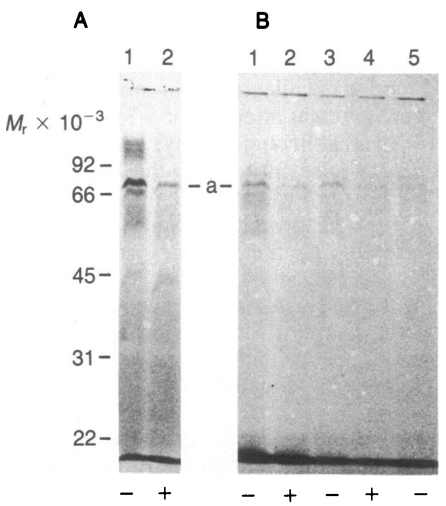

FIG. 2. NaDodSO$_4$/polyacrylamide gel electrophoresis of vacuolar membranes labeled with [^{14}C]MalNEt (*A*) and [^{14}C]NBD-Cl (*B*). Vacuolar membranes were incubated with inhibitors in the presence (+) or absence (−) of 5 mM MgADP (*A*) or MgATP (*B*). In *B*, incubation mixtures contained either 0.25 μCi (lanes 1, 2, and 5) or 0.12 μCi (lanes 3 and 4) of [^{14}C]NBD-Cl. The mixture in lane 5 (*B*) also contained 100 mM KSCN. The line at "a" indicates the M_r ≈70,000 polypeptide.

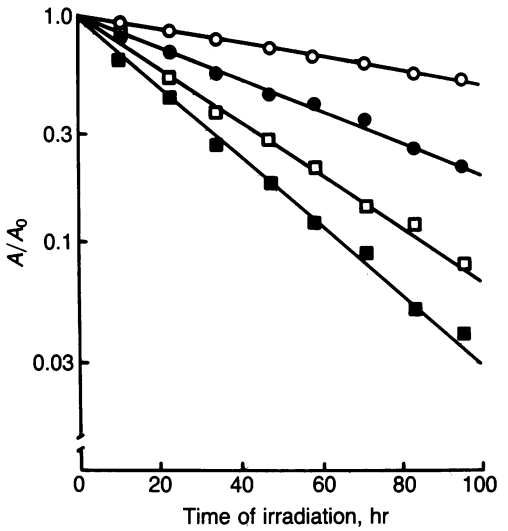

FIG. 3. Comparison of the functional sizes of vacuolar and mitochondrial ATPases estimated by radiation inactivation. Membranes were exposed to irradiation by ^{60}Co and assayed for loss of ATPase activity. ATPase activities of vacuolar and mitochondrial membranes were plotted as the KSCN-sensitive and KN_3-sensitive activities, respectively. (Without the inhibitors the calculated sizes were 10% smaller for both ATPases.) Analysis of the data by linear regression showed that inactivation to 37% of the level of the unirradiated control occurred after 137 hr for glucose-6-phosphate dehydrogenase (○) (extrapolated value), after 61 hr for Ca^{2+}-ATPase (●), after 37 hr for the mitochondrial ATPase (□), and after 29 hr for the vacuolar ATPase (■).

fer blot analysis to test for immunological crossreactivity. Antibody to the plasma membrane ATPase reacted strongly only with the plasma membrane fraction (Fig. 4*A*). In other fractions, there was slight reactivity with a M_r 104,000 polypeptide, which was presumably due to contamination by plasma membrane ATPase. Similarly, antibody to the *E. coli* F_1 ATPase or its β subunit was specific for the mitochondrial ATPase β subunit (Fig. 4*B*), which is characteristically conserved among F_0F_1 ATPases (12). No crossreaction was detected with the vacuolar membrane ATPase, indicating that it belonged to a third immunologically distinct group of enzymes.

Comparison with the Tonoplast ATPase from Corn. An ATPase with properties and components very similar to those of the *Neurospora* vacuolar membrane has been isolated from tonoplasts of corn coleoptiles (10). We compared corn tonoplasts, *Neurospora* vacuolar membranes, and the partially purified ATPases from both by $NaDodSO_4$ gel electrophoresis (Fig. 5). The most striking observation was that, although the patterns of polypeptides in the two membranes were clearly different, the partially purified ATPases appeared very similar. The most prominent bands in both ATPase preparations were polypeptides of M_r ≈70,000 and ≈62,000.

Polyclonal antibodies were raised against the two major polypeptides of the partially purified corn tonoplast ATPase, and they were tested for crossreactivity with *Neurospora* membrane fractions and partially purified ATPases. Antibody to the M_r ≈70,000 (10) polypeptide of corn tonoplasts showed no binding to plasma membrane and mitochondrial ATPases of *Neurospora* but bound strongly and specifically to the M_r ≈70,000 polypeptide of the partially purified vacuolar membrane ATPase (Fig. 6). In vacuolar membranes, the mobility of this polypeptide was altered, apparently by glycoproteins found in the M_r 50,000–60,000 region of the gel (unpublished results). (When vacuolar membranes were mixed with other fractions, they caused a similar displacement of high molecular weight proteins, as illustrated in Fig. 4*B*, lane 3.) Antibody to the M_r 62,000 polypeptide from the corn tonoplast ATPase did not crossreact with any of the *Neurospora* fractions (data not shown).

DISCUSSION

Three H^+-translocating ATPases have been identified in *Neurospora*. Of these, two have been purified and well characterized. The plasma membrane ATPase appears to be a dimer of identical polypeptides of M_r 104,000 and belongs to the E_1E_2 family of ATPases (15, 20). The mitochondrial ATPase has a much more complex structure, consisting of ≈11 different polypeptides, and is typical of the F_0F_1 category (21). The data in this paper suggest that the ATPase in the vacuolar membranes belongs to a third group of

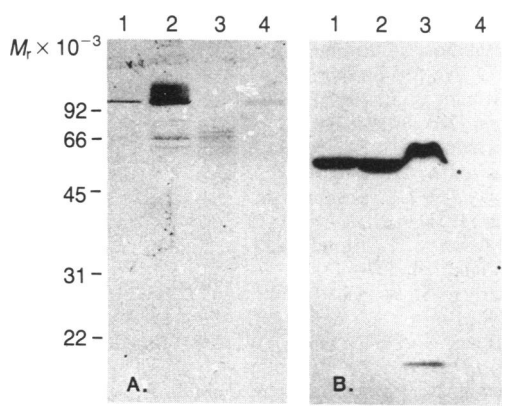

FIG. 4. Immunological distinctiveness of *Neurospora* H^+-ATPases. Immunoblotting was performed with rabbit antisera and ^{125}I-labeled protein A. (*A*) Antiserum to the *Neurospora* plasma membrane ATPase. Lane 1, mitochondria, 6 μg; lane 2, plasma membranes, 6 μg; lane 3, vacuolar membranes, 6 μg; lane 4, vacuolar membrane ATPase, 60 μl of enzyme partially purified as in ref. 3. (*B*) Antiserum to the β subunit of the *E. coli* F_0F_1 ATPase. Lane 1, partially purified mitochondrial ATPase, 10 μg; lane 2, mitochondria, 40 μg; lane 3, mitochondria and vacuolar membranes, 30 μg each; lane 4, vacuolar membranes, 40 μg.

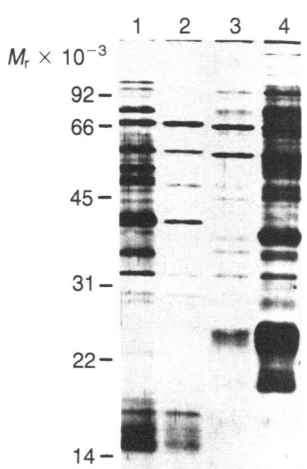

FIG. 5. Comparison of ATPase from *N. crassa* and corn. Membranes (6 μg of protein) and partially purified ATPases (2 μg of protein) from *Neurospora* vacuoles and corn tonoplasts were electrophoresed on $NaDodSO_4$/polyacrylamide gel (12%) and stained with silver. Lane 1, *Neurospora* vacuolar membranes; lane 2, *Neurospora* ATPase; lane 3, corn ATPase; lane 4, tonoplast membranes.

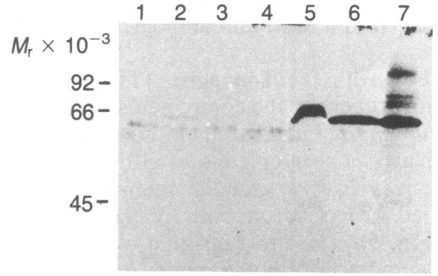

FIG. 6. Immunological crossreactivity between ATPases of *Neurospora* vacuoles and corn tonoplasts. Immunoblotting was performed with rabbit antiserum to the M_r ≈70,000 protein of corn tonoplast membranes and ^{125}I-labeled protein A. Lane 1, molecular weight markers; lane 2, plasma membranes, 50 μg; lane 3, mitochondria, 50 μg; lane 4, partially purified mitochondrial ATPase, 20 μg; lane 5, vacuolar membranes, 50 μg; lane 6, partially purified vacuolar membrane ATPase, 80 μl of Triton X-100-solubilized, gradient-purified enzyme; lane 7, corn tonoplast membranes, 15 μg.

ATPases—a group that appears to be a variation of the F_0F_1 type.

Partial purification of the vacuolar membrane ATPase strongly implicated two polypeptides, of M_r ≈70,000 and ≈62,000, as major components. These two proteins consistently copurified with ATPase activity in an approximate ratio (by staining intensity) of 2:1. Furthermore, the M_r ≈70,000 polypeptide was preferentially labeled in an adenylate-protectable manner by two inhibitors of vacuolar membrane ATPase activity, NBD-Cl and MalNEt. NBD-Cl also preferentially labels the β subunit, which contains the active site, of mitochondrial ATPases (18, 19). Several polypeptides of lower molecular weight and lesser amount (by staining) also copurified with activity and might be components of the ATPase. In particular, a band of M_r ≈15,000 was labeled by [^{14}C]DCCD in vacuolar membranes and partially purified vacuolar ATPase.

These results suggest that the vacuolar membrane ATPase, like the mitochondrial ATPase, is an oligomeric complex of several different types of polypeptides. Measurements of the functional sizes of the enzymes are consistent with this idea. The size of the mitochondrial ATPase, obtained by radiation inactivation, was M_r ≈4.6 × 10^5 [compared with a predicted value of M_r 5.3 × 10^5 obtained by adding the molecular weights of all subunits (21)]. The value for the vacuolar membrane ATPase was about 15% greater. By contrast, the plasma membrane ATPase of *Neurospora* has a much smaller functional size of 2.3 × 10^5 daltons (15).

Direct comparison of the vacuolar membrane ATPases from *Neurospora* and corn supports the hypothesis that these enzymes represent a third class of ATPases. The corn enzyme also has a large functional size (10), and partially purified ATPases from both sources are composed predominantly of M_r ≈70,000 and ≈62,000 polypeptides. In corn, these two polypeptides copurified in a 1:1 ratio. We cannot as yet determine if *Neurospora* and corn actually have different stoichiometries for these polypeptides or if we are merely observing differential staining. Both ATPases appear to have a small DCCD-binding polypeptide (2, 22). Most strikingly, antibody to the M_r ≈70,000 polypeptide of corn crossreacts with a polypeptide of the same size in *Neurospora*, whereas antibody to the M_r ≈62,000 protein does not crossreact. This last observation is reminiscent of results obtained with F_0F_1 ATPases. Crossreaction is found among β subunits from mitochondria and *E. coli* (Fig. 4*B*), as well as chloroplasts (12), but not among α subunits (12). To our knowledge, published data on the subunit composition of other organellar ATPases are presently available only for yeast (9) and corn (10) vacuoles. As further reports of purification and antibody preparation appear, the comparative approach employed in this paper can serve as a further test of the hypothesis that these enzymes belong to a third group of ATPases.

Although there is no evidence for an easily removable F_1 portion in the vacuolar ATPases of *Neurospora* or corn, they both resemble closely an F_0F_1 ATPase purified from the anaerobic bacterium *Clostridium pasteurianum*. This enzyme contains four subunits of M_r 66,000 (labeled by [^{14}C]NBD-Cl), 58,000, 43,000, and 15,000 (labeled by [^{14}C]DCCD) in a probable stoichiometry of 2:1:2:8, giving a calculated M_r for the complex of ≈400,000 (23). Maloney and Wilson (24) have speculated that the *Clostridium* ATPase may represent an early stage in the evolution of all ion-translocating ATPases. Perhaps corn and *Neurospora* vacuoles have preserved that early prokaryotic form of ATPase.

This work was supported by grants from the National Institutes of Health (GM-28703) and the National Science Foundation (PCM-8301995).

1. Vaughn, L. E. & Davis, R. H. (1981) *Mol. Cell. Biol.* **1**, 797–806.
2. Bowman, E. J. (1983) *J. Biol. Chem.* **258**, 15238–15244.
3. Bowman, E. J. & Bowman, B. J. (1985) in *Biochemistry and Function of Vacuolar Adenosine-Triphosphatase in Fungi and Plants*, ed. Marin, B. P. (Springer, Berlin), pp. 131–140.
4. Sze, H. (1985) *Annu. Rev. Plant Physiol.* **36**, 175–208.
5. Kakinuma, Y., Ohsumi, Y. & Anraku, Y. (1981) *J. Biol. Chem.* **256**, 10859–10863.
6. Njus, D., Knoth, J. & Zallakian, M. (1981) *Curr. Top. Bioenerg.* **11**, 107–147.
7. Schneider, D. L. (1977) *J. Membr. Biol.* **34**, 247–261.
8. Forgac, M., Cantley, L., Wiedenmann, B., Altstiel, L. & Branton, D. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 1300–1303.
9. Uchida, E., Ohsumi, Y. & Anraku, Y. (1985) *J. Biol. Chem.* **260**, 1090–1095.
10. Mandala, S. & Taiz, L. (1985) *Plant Physiol.* **78**, 327–333.
11. Bowman, E. J., Bowman, B. J. & Slayman, C. W. (1981) *J. Biol. Chem.* **256**, 12336–12342.
12. Rott, R. & Nelson, N. (1981) *J. Biol. Chem.* **256**, 9224–9228.
13. Brooker, R. J. & Slayman, C. W. (1983) *J. Biol. Chem.* **258**, 222–226.
14. Vaitukaitis, J., Robbins, J. B., Nieschlag, E. & Ross, G. T. (1971) *J. Clin. Endocrinol.* **33**, 988–991.
15. Bowman, B. J., Berenski, C. J. & Jung, C. Y. (1985) *J. Biol. Chem.* **260**, 8726–8730.
16. Bowman, B. J., Blasco, F. & Slayman, C. W. (1981) *J. Biol. Chem.* **256**, 12343–12349.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
18. Ferguson, S. J., Lloyd, W. J. & Radda, G. K. (1975) *Eur. J. Biochem.* **54**, 127–133.
19. Esch, F. S. & Allison, W. S. (1979) *J. Biol. Chem.* **254**, 10740–10746.
20. Goffeau, A. & Slayman, C. W. (1981) *Biochim. Biophys. Acta* **639**, 197–223.
21. Ludwig, B., Prochaska, L. & Capaldi, R. A. (1980) *Biochemistry* **19**, 1516–1523.
22. Mandala, S. (1985) Dissertation (Univ. of California, Santa Cruz).
23. Clarke, D. J., Fuller, F. M. & Morris, J. G. (1979) *Eur. J. Biochem.* **98**, 597–612.
24. Maloney, P. C. & Wilson, T. H. (1985) *BioScience* **35**, 43–48.