JOURNAL OF BACTERIOLOGY, Sept. 1982, p. 1326–1337
0021-9193/82/091326-12$02.00/0

Vol. 151, No. 3

Identification and Properties of an ATPase in Vacuolar Membranes of *Neurospora crassa*

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Received 9 February 1982/Accepted 11 May 1982

Using a vacuolar preparation virtually free of contamination by other organelles, we isolated vacuolar membranes and demonstrated that they contain an ATPase. Sucrose density gradient profiles of vacuolar membranes show a single peak of ATPase activity at a density of 1.11 g/cm^3. Comparison of this enzyme with the two well-studied proton-pumping ATPases of *Neurospora* plasma membranes and mitochondria shows that it is clearly distinct. The vacuolar membrane ATPase is insensitive to the inhibitors oligomycin, azide, and vanadate, but sensitive to *N,N'*-dicyclohexylcarbodiimide (K_i = 2 μM). It has a pH optimum of 7.5, requires a divalent cation (Mg^{2+} or Mn^{2+}) for activity, and is remarkably unaffected (±20%) by a number of monovalent cations, anions, and buffers. In its substrate affinity (K_m for ATP = 0.2 mM), substrate preference (ATP > GTP, ITP > UTP > CTP), and loss of activity with repeated 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid washes, the vacuolar membrane ATPase resembles the F_1F_0 type of ATPase found in mitochondria and differs from the integral membrane type of ATPase in plasma membranes.

The simple eucaryotic organism *Neurospora crassa* contains, in addition to other organelles, small vacuoles that can be identified by their contents of basic amino acids, polyphosphate, and hydrolytic enzymes (28, 45, 47). Like yeast vacuoles (49), plant vacuoles (4, 31), and animal lysosomes (14), *Neurospora* vacuoles are postulated to function in both metabolite storage and intracellular digestion.

A well-established role of *Neurospora* vacuoles is that they constitute intracellular compartments where arginine and other basic amino acids are sequestered (13). During growth on minimal medium, over 98% of the arginine of the cell is stored inside the vacuole. Consequently, cytoplasmic levels of arginine are rather low (approximately 0.2 mM), sufficient to maintain protein synthesis but too low to support degradation of arginine through the catabolic enzyme arginase (48). That the stored arginine remains accessible as a nitrogen source is shown by the rapid mobilization of the vacuolar contents when cells are transferred to a medium lacking nitrogen (24).

Although the mechanism for maintaining large concentration gradients of arginine between the vacuole and cytoplasm is not known, two hypotheses have been advanced. First, it has been suggested that in the yeast *Saccharomyces cerevisiae*, which has a basic amino acid storage vacuole similar to that of *N. crassa*, basic amino acids are held inside vacuoles by binding to polyphosphate (17). Under most growth conditions, vacuoles of *S. cerevisiae* and *N. crassa* contain approximately equal amounts of polyphosphate and basic amino acids. Recent experiments with *N. crassa*, however, have shown that if cells are grown under conditions of phosphate starvation, vacuolar levels of polyphosphate may drop by 90% with no effect on vacuolar arginine levels (11). Thus, polyphosphate has no obligatory role in arginine accumulation in vacuoles, at least in *N. crassa*.

The second hypothesis is that the energy needed for arginine transport is provided by a proton-translocating ATPase. Several recent results with *S. cerevisiae* lend strong support to this hypothesis. Ohsumi and Anraku (33) succeeded in demonstrating that arginine accumulation into vacuolar membrane vesicles of *S. cerevisiae* requires ATP as an energy source. Furthermore, the ATP-driven uptake is inhibited by *N,N'*-dicyclohexylcarbodiimide (DCCD), an inhibitor of proton translocation, and by 3,5-di-*tert*-butyl-4-hydroxybenzilidenemalononitrile (SF6847), a protonophore uncoupler. The proposed mechanism for arginine accumulation in vacuolar membrane vesicles is an H^+/arginine antiport driven by a proton motive force generated by a vacuolar membrane ATPase. Recent-

ly, the properties of an H^+-translocating ATPase in these vacuolar membranes have been described (21).

In *N. crassa*, energy-dependent transport of arginine by isolated vacuoles or vacuolar membrane vesicles has not been demonstrated. However, whole-cell studies by Drainas and Weiss (15) led to the conclusion that metabolic energy is required for arginine accumulation into vacuoles, consistent with the role of a proton-pumping ATPase as proposed for *S. cerevisiae*.

In the work reported here, we used a highly purified vacuolar preparation (11, 45) to prepare vacuolar membranes. We demonstrated the presence of a DCCD-sensitive Mg^{2+}-ATPase in these membranes. This ATPase is distinguishable by several criteria from plasma membrane and mitochondrial ATPases, both well-characterized proton-pumping ATPases in *N. crassa* (5, 7, 9, 20, 26, 38). The characteristics of the vacuolar enzyme suggest a close similarity to membrane-bound ATPases of chromaffin granules (1, 2), lysosomes (39), and plant vacuoles (12).

MATERIALS AND METHODS

Growth of cells. Wild-type *N. crassa* RL21a was used throughout. Conidia 6 to 14 days old were inoculated at a density of 10^6/ml into 4 liters of Vogel minimal medium (46) containing 2% sucrose and then were grown with vigorous aeration at 25°C for 15 h. The mycelial pad, collected by filtration through cheesecloth, weighed approximately 25 g (wet weight).

Isolation of vacuoles. (i) Method A. The procedures of Cramer et al. (11) for the isolation of vacuoles were used with some modifications. Cells from a 4-liter culture were divided into three equal aliquots, and each was suspended with vigorous shaking in 45 ml of snail enzyme medium containing 0.6 M sorbitol, 1 mM EDTA, 0.14 M β-mercaptoethanol, and 10 mM citric acid, adjusted to pH 5.8 with K_2HPO_4. After the addition of 200 mg of β-glucuronidase (type H-1) predissolved in 5 ml of snail enzyme medium to each aliquot, the cells were digested for 30 min at 30°C with occasional gentle shaking. Cells were harvested by centrifugation for 7 min at 750 × *g* in a Sorvall SS34 rotor and were then washed twice by suspending in 120 ml of 1 M sorbitol and centrifuging for 7 min at 750 × *g*. Washed, snail enzyme-weakened cells were suspended in 75 ml of vacuolar preparation medium (1 M sorbitol–1 mM EDTA–10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], adjusted to pH 7.5 with NaOH) and homogenized in a glass homogenizer with six slow passes of a smooth-bottomed Teflon pestle. The homogenate was centrifuged for 6 min at 750 × *g*, yielding a cell pellet and a crude homogenate, or S750 fraction. The cell pellet was extracted twice more by suspension in 60 ml of vacuolar preparation medium and homogenization with six strokes. The homogenate was centrifuged for 10 min at 750 × *g*. The supernatants from three 750 × *g* spins were pooled, yielding approximately 200 ml of the S750 fraction, and filtered through two layers of Miracloth. In later experiments, filtration was through glass microfiber filter paper (Whatman grade 934-AH, 12.5-cm diameter) placed on a fritted plastic support (Bel-Art Products). The filtrate was centrifuged for 20 min at 14,500 × *g*, yielding a crude mitochondrial pellet, or P15,000 fraction, and a supernatant fraction (S15,000 fraction) which was routinely discarded. In addition to mitochondria, the P15,000 fraction also contained vacuoles. The P15,000 fraction was suspended in vacuolar preparation medium to a final volume of 2.0 to 2.5 ml and loaded on three 4.6-ml linear sucrose gradients (30 to 60% [wt/wt] sucrose in 1 mM EDTA–10 mM HEPES-NaOH buffer, pH 7.5). The gradients were centrifuged for 3 h at 100,000 × *g* (R_{avg}). The gradients contained two light bands toward the top, a large band of mitochondria approximately one-third into the gradient, and a small orange-brown pellet of vacuoles. The supernatants were aspirated off, and the pellet was suspended in 1 to 2 ml of vacuolar preparation medium, frozen in dry ice-acetone, and stored in aliquots at −70°C. Alternatively, the vacuolar pellets were used immediately to isolate vacuolar membranes.

(ii) Method B. The P15,000 (or mitochondrial) fraction was isolated from a 12-liter culture as described previously (9). Method B was basically similar to method A and differed mainly in the use of 0.33 M sucrose rather than 1.0 M sorbitol as the osmoticum in vacuolar preparation medium. A vacuolar pellet was obtained from the P15,000 fraction by centrifugation for 4 h at 85,000 × *g* through a 30-ml, 30 to 60% linear sucrose density gradient.

Isolation of vacuolar membranes and ultracentrifuge gradient analysis. Vacuoles prepared by either method A or method B described above were osmotically lysed by direct suspension into 15 ml of 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA; adjusted to pH 7.5 with Tris base) and homogenized vigorously in a glass homogenizer with 10 strokes of a smooth-bottomed Teflon pestle. A vacuolar membrane fraction was collected by centrifugation for 1 h at 100,000 × *g*. The membrane pellets were suspended in 1.0 to 1.2 ml of 1 mM EGTA–Tris by hand homogenization in a small glass homogenizer, frozen in a dry ice-acetone bath, and stored in aliquots at −70°C. Enzymatic and chemical determinations of vacuolar membrane contents were carried out on aliquots subjected to a single freeze-thaw cycle.

In some experiments, vacuolar membrane fractions were analyzed on linear sucrose density gradients. The 4.6-ml gradients of 15 to 45% (wt/wt) sucrose in 1 mM EDTA–10 mM HEPES-NaOH buffer, pH 7.5, were loaded with membrane fraction and centrifuged for 3 h at 170,000 × *g* (R_{avg}). Fractions were collected with a Buchler gradient eluter and analyzed for enzymatic activities as described below. Density was determined by refractometry.

Vacuolar membrane ATPase assay. In most cases, vacuolar membrane ATPase was assayed colorimetrically by the release of P_i from ATP. The standard reaction mixture consisted of 5 mM Na_2ATP, 5 mM $MgCl_2$, 10 mM NH_4Cl, 5 mM KN_3 (to inhibit residual mitochondrial ATPase), 20 μM Na_3VO_4 (to inhibit residual plasma membrane ATPase), and 10 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) buffer adjusted to pH 7.5 with Tris base. The addition of 3 to 6 μg of membrane protein to 500 μl of reaction mixture

initiated the assay. After 60 min (or when 3 to 6% of the ATP was hydrolyzed) at 30°C, the reaction was stopped with 100 μl of 50% trichloroacetic acid and assayed for P_i by the method of Dryer et al. (16). Additions to or deviations from the standard reaction mixture are indicated in table footnotes and figure legends where appropriate. In experiments in which inhibition by DCCD was examined, DCCD (from a 0.2 M stock solution in 100% ethanol) was added with rapid mixing to the enzyme in the absence of a substrate to allow binding, and the reaction was initiated with a substrate. In some cases, vacuolar membrane ATPase was assayed by the release of $^{32}P_i$ from [γ-^{32}P]ATP as described previously (8).

Other enzyme assays. To assess the contamination of vacuolar membrane ATPase by mitochondrial and plasma membrane ATPases, the standard reaction mixture for vacuolar membrane ATPase was altered in accordance with distinguishing characteristics of pH optima and inhibitor sensitivities of these two enzymes (6). Mitochondrial ATPase was assayed as the azide-sensitive or oligomycin-sensitive activity in a mixture of 5 mM Na_2ATP, 5 mM $MgCl_2$, 10 mM NH_4Cl, and 10 mM PIPES buffer, adjusted to pH 8.3 with Tris base, in the presence and absence of 5 mM KN_3 or 10 μg of oligomycin per ml. Plasma membrane ATPase was assayed as the vanadate-sensitive activity in a mixture of 5 mM Na_2ATP, 5 mM $MgCl_2$, 10 mM NH_4Cl, 5 mM KN_3, and 10 mm PIPES buffer, adjusted to pH 6.7 with Tris base, in the presence and absence of 20 μM Na_3VO_4. After 60 min at 30°C, the reaction was stopped with trichloroacetic acid, and P_i was assayed colorimetrically as above. Even though activity of both these ATPases is improved in the presence of a recycling system including pyruvate kinase and phosphoenolpyruvate (6), no recycling system was used in these experiments because vacuoles and vacuolar membranes contain phosphatase activity which cleaves phosphoenolpyruvate.

Phosphatase activity was assessed by the hydrolysis of 5 mM *p*-nitrophenylphosphate (added in place of ATP) in reaction mixtures used for mitochondrial, plasma membrane, and vacuolar ATPase determinations. No activity was seen in the absence of $MgCl_2$. Usually the reaction was stopped with KOH-ethanol, and the amount of *p*-nitrophenol was determined by the absorbance at 405 nm (A_{405}) compared with a standard curve of *p*-nitrophenol by published procedures (10). Alternatively, the reaction was stopped with trichloroacetic acid, and P_i was measured colorimetrically as for ATPase assays.

AMPase activity was determined as P_i released from AMP in vacuolar membrane ATPase standard reaction mixture at pH 7.5 with AMP in place of ATP. Omission of $MgCl_2$ had no effect on AMPase activity.

α-Mannosidase was measured at 30°C by the procedure of van der Wilden and Matile (43). Specific activities were calculated by using a molar extinction coefficient of 1.83×10^4 for *p*-nitrophenol in 0.1 M Na_2CO_3 at 400 nm (19).

Protease activity was determined at 30°C in a 500-μl total volume as the increase in A_{595} when enzyme fractions were incubated with 2 mg of hide powder azure suspended in 5 mM KN_3–0.1 M Tris-hydrochloride buffer, pH 7.8. The reaction was stopped with 2 ml of 10% trichloroacetic acid. Samples were centrifuged, and the supernatant was read at A_{595}.

Succinate dehydrogenase was assayed by the method of Pennington (36) in the presence of 0.1% bovine serum albumin (BSA) as recommended by King (22).

Estimation of protein and arginine. Samples were suspended in 0.4% sodium deoxycholate, and protein was estimated by the procedure of Lowry et al. (25) with BSA as the standard. Arginine (soluble, nonprotein) was estimated in cold trichloroacetic acid extracts. Samples were incubated in 4.3% trichloroacetic acid for 20 min in an ice bath and centrifuged. A 500-μl sample was neutralized with 500 μl of 0.5 N NaOH and assayed for arginine by the procedure of van Pilsum et al. (44).

Reagents. Most chemicals were obtained from Sigma Chemical Co., St. Louis, Mo., including β-glucuronidase (type H-1), BSA (fraction V powder), oligomycin, phenylmethylsulfonyl fluoride (PMSF), all nucleoside phosphates, *p*-nitrophenylphosphate, *p*-nitrophenyl-α,D-mannopyranoside, phosphoenlopyruvate (monopotassium salt), PIPES, HEPES, EDTA, and EGTA. Hide Powder Azure was purchased from Calbiochem, La Jolla, Calif. Potassium azide and *N*-phenyl-*p*-phenylenediamine hydrochloride (semidine) came from Eastman Kodak Co., Rochester, N.Y. Sodium vanadate was from Fisher Scientific Co., Pittsburgh, Pa. DCCD was purchased from Aldrich Chemical Co., Milwaukee, Wis. [γ-^{32}P]ATP (specific activity, 2,000 Ci/mmol) was from Amersham Corp., Arlington Heights, Ill. Complete counting cocktail 3a70B came from Research Products International Corp., Elk Grove Village, Ill.

RESULTS

Isolation of vacuoles. Using a modified version of the procedure of Cramer et al. (11) (method A above), we were able to isolate a highly purified vacuolar fraction. Our primary vacuolar marker was the ratio of sedimentable (nonprotein) arginine to protein, which monitors only intact vacuoles and avoids problems such as multiple locations and latency which are typical of enzymatic markers. In addition, we followed the distribution of α-mannosidase and protease, two enzymes primarily localized in vacuoles of both *N. crassa* and *S. cerevisiae* (45, 49), and AMPase, an activity that is highly enriched in yeast vacuoles (49).

Table 1 shows the distribution of protein and vacuolar markers in a typical cell fractionation experiment. Of the 960 mg of protein in the crude homogenate (the S750 fraction), 132 mg (14%) was recovered in the low-speed pellet (P15,000 fraction), or crude organellar fraction. In the next step, this fraction was layered on a 30 to 60% linear sucrose density gradient and yielded a large band of mitochondria about one-third down the gradient and a vacuolar pellet containing 2.9 mg, or 2%, of the protein from the P15,000 fraction. Sedimentable arginine showed a pattern quite different from that of protein. From 67 μmol of arginine in the S750 fraction, 32 μmol (48%) sedimented in the P15,000 fraction, and of this, 11 μmol (34%) sedimented through

the sucrose gradient to the vacuolar pellet. Thus, by this criterion we calculated a 3.4-fold enrichment of vacuoles in the P15,000 fraction and a further 15-fold purification on the sucrose gradient.

The three enzymatic markers, α-mannosidase, AMPase, and protease, qualitatively copurified with arginine, as shown by their moderate enrichment in the P15,000 fraction and considerable further enrichment in the vacuolar fraction. Quantitatively, small differences were seen among the three enzymatic activities. AMPase activity showed excellent copurification with arginine, with a 3.3-fold enrichment in the P15,000 fraction and a further 15-fold enrichment in the vacuolar fraction, and is therefore likely localized in vacuoles of *N. crassa*. Once sedimented, α-mannosidase activity copurified with arginine, as shown by the 15-fold enrichment between the P15,000 and vacuolar fractions. Since the sedimentability of α-mannosidase activity from the S750 fraction to the P15,000 fraction was 36%, or 12% less than arginine, a portion of this activity may have a nonvacuolar location in *N. crassa*. (Rat liver preparations contain three distinct α-mannosidases located in lysosomes, the Golgi apparatus, and the cytosol [34].) The lack of quantitative copurification between protease activity and arginine may result from the presence of cytoplasmic protease inhibitors (51). Two observations, the low specific activities of protease in the S750 and S15,000 fractions, and the 662% recovery of protease activity in the P15,000 fraction, suggested this explanation.

As reported previously (45), vacuoles were virtually free of mitochondrial contamination. The specific activity of succinate dehydrogenase in the P15,000 fraction of Table 1 was 11.1 nmol/min per mg of protein (and up to 26 nmol/min per mg of protein in other experiments), yet no activity was detected in the vacuolar fraction (data not shown).

In conclusion, the purified vacuoles, isolated as a pellet in a sucrose gradient with a maximum density of 1.28 g/cm^3, showed a 54-fold enrichment from the crude homogenate and contained no detectable mitochondrial contamination as measured by succinate dehydrogenase.

Isolation of vacuoles by method B above resulted in lower vacuolar yields per liter of cells. However, no differences in vacuolar quality were observed.

Isolation of vacuolar membranes. From highly purified vacuoles we isolated a membrane fraction. Severe osmotic shock was employed to lyse the vacuoles and to release soluble contents, and a pellet was collected by centrifugation. By this procedure we obtained a membrane fraction containing 17% of the vacuolar protein

TABLE 1. Distribution of vacuolar markers during cell fractionation[a]

Fraction	Protein (mg)	Arginine		α-Mannosidase		AMPase		Protease	
		Sp act (μmol/mg)	Units (μmol/min)	Sp act (nmol/min per mg)	Units (nmol/min)	Sp act (μmol/min per mg)	Units (μmol/min)	Sp act (ΔA_{595}/min per mg)	Units (ΔA_{595}/min)
S750	960	0.07	67	0.17	163	0.03	29	0.0008	0.8
S15,000	819	0.04	33	0.13	106	0.02	16	0.001	0.8
P15,000	132	0.24	32	0.45	59	0.10	13	0.04	5.3
Vacuoles	2.9	3.79	11	6.97	20	1.48	4.3	0.28	0.8
Vacuolar membranes	0.5	0.21	0.1	11.1	5.6	0.95	0.5	0.42	0.2

[a] Procedures for cell fractionation and marker assays are described in the text. An 8-liter culture was used.

and no more than 1% of vacuolar arginine (Table 1). The majority (72 to 88%) of α-mannosidase, AMPase, and protease activities was not recovered in the pellet, suggesting a soluble form in vacuoles. However, significant amounts of all three marker enzymes did remain associated with the membranes, and both α-mannosidase and protease showed modest increases (1.6- and 1.5-fold, respectively) in specific activities in the membrane fraction (Table 1). Whether these enzymes are loosely associated or in part tightly bound to the vacuolar membrane in *N. crassa* remains to be determined.

Identification of ATPase activity in vacuolar membranes. As a test of the hypothesis that vacuolar membranes contain an ATP-driven proton pump, we assayed the ability of vacuoles and vacuolar membranes to hydrolyze ATP. A typical vacuolar fraction hydrolyzed ATP with specific activities of 0.13 and 0.20 μmol/min per mg of protein at pH 6.7 and 8.3, respectively (Table 2). Vacuolar membranes prepared from these vacuoles showed approximately fivefold-higher specific activities of 0.71 and 1.07 μmol/min per mg of protein at pH 6.7 and 8.3, respectively. These findings, together with the observation that 20% of the protein from whole vacuoles was recovered in vacuolar membranes (Table 1), suggested the presence of a vacuolar membrane ATPase.

Before it could be concluded that the vacuolar membranes possessed a unique kind of ATPase activity, two possible alternative explanations had to be ruled out: contamination with other membranes known to contain ATPases (especially plasma membranes and mitochondria) and nonspecific hydrolysis of ATP by phosphatases known to be present in vacuolar preparations (28, 45). The experiment shown in Table 2 was designed to address both of these possibilities.

TABLE 2. Identification of a distinct ATPase in vacuolar membranes[a]

Assay conditions	Activity (μmol/min per mg)			
	ATPase		Phosphatase	
	Vacuoles	Vacuolar membranes	Vacuoles	Vacuolar membranes
pH 6.7, azide	0.13	0.71	0.09	0.11
pH 6.7, azide, vanadate	0.10	0.61	0.07	0.07
pH 8.3	0.20	1.07	0.47	0.57
pH 8.3, azide	0.16	1.02	0.47	0.66
pH 8.3, azide, vanadate	0.17	0.72	0.21	0.21

[a] The reaction mixtures contained 5 mM substrate (ATP or *p*-nitrophenylphosphate), 5 mM $MgCl_2$, 10 mM NH_4Cl, and 10 mM PIPES, adjusted to pH 6.7 or 8.3 with Tris base. Additions included 5 mM KN_3 and 20 μM Na_3VO_4. Activity was assayed as described in the text.

The best way to recognize plasma membrane and mitochondrial ATPases is by their sensitivity to vanadate at pH 6.7 and to azide at pH 8.3 (6). When vacuolar membranes were assayed at pH 6.7 in the presence of 5 mM KN_3 to inhibit residual mitochondrial ATPase, vanadate (20 μM) inhibited ATP hydrolysis by only 14% (Table 2). From the assumption that all of the vanadate-sensitive activity (0.1 μmol/min per mg of protein) was due to plasma membrane ATPase, together with the finding that purified plasma membranes have an activity of 7.0 μmol/min per mg of protein (9), we calculated that 1.4% of the protein in the vacuolar membrane preparation might have come from contamination by plasma membranes. At pH 8.3, azide (5 mM) inhibited ATP hydrolysis by only 5%. As above, from the assumptions that all of the azide-sensitive activity (0.05 μmol/min per mg of protein) was due to mitochondrial contamination and that mitochondrial membranes have an activity of 2.4 μmol/min per mg of protein (26), we calculated that 2.1% of the protein in the vacuolar membrane fraction might be due to contamination by mitochondrial membranes. Taken together, these data showed that approximately 80% of ATP hydrolysis by the vacuolar membrane preparation was insensitive to vanadate and azide, could not be ascribed to plasma membrane or mitochondrial ATPase, and therefore appeared to be catalyzed by an enzyme on vacuolar membranes.

Because ATP hydrolysis could be due to a nonspecific vacuolar phosphatase, we also measured hydrolysis of *p*-nitrophenylphosphate as a probe for phosphatase activity (Table 2). At pH 6.7 the specific activity of phosphatase in vacuoles was quite low (0.09 μmol/min per mg of protein) and, in contrast to ATPase activity, showed little increase in the vacuolar membrane preparation (0.11 μmol/min per mg of protein). At pH 8.3, rates of *p*-nitrophenylphosphate hydrolysis were fivefold higher than at pH 6.7, but again, activity in vacuolar membranes (0.66 μmol/min per mg of protein) was not significantly enriched over that in whole vacuoles (0.47 μmol/min per mg of protein). The specific activity of *p*-nitrophenylphosphate hydrolysis continued to increase up to at least pH 9 (data not shown), indicating that indeed alkaline phosphatase was associated with the vacuolar membranes. In further contrast to ATP hydrolysis, *p*-nitrophenylphosphate hydrolysis was inhibited 68% by 20 μM vanadate (pH 8.3), a second indication of the presence of alkaline phosphatase (41). Alkaline phosphatase might account for a portion of ATP hydrolysis at pH 8.3, the 0.3 μmol/min per mg of protein which was

sensitive to vanadate. However, four characteristics of the phosphatase activity, relatively low rates of *p*-nitrophenylphosphate hydrolysis (especially at pH 6.7), strong alkaline pH dependence, vanadate sensitivity, and lack of enrichment in vacuolar membranes, clearly differed from the ATPase activity. Thus, we concluded that the majority of ATP hydrolysis in vacuolar membrane preparations was catalyzed not by a phosphatase, but by a specific ATPase in vacuolar membranes. Further support for this conclusion was provided by experiments described below.

Assay for vacuolar membrane ATPase. For routine assays of the vacuolar membrane ATPase, we included 5 mM azide and 20 μM vanadate in the reaction mixture. The pH optimum of the ATPase was determined over a range from pH 3.2 to 8.9 and revealed a broad optimum between pH 6.5 and 8.0. Activity was maximal at approximately pH 7.5, and this pH was used in all subsequent assays.

By using [γ-^{32}P]ATP as the substrate, we verified that essentially 100% of the vacuolar membrane ATPase activity was due to cleavage of the terminal phosphate of ATP under these assay conditions. In five experiments in which ATPase specific activity varied from 0.22 to 0.63 μmol/min per mg of protein, the amount of phosphate released as $^{32}P_i$ ranged from 88 to 108% of that obtained by the colorimetric assay for total P_i.

Membranous localization of ATPase activity. As indicated above, most of the ATPase activity in whole vacuoles was recovered in the vacuolar membrane preparation with a fivefold increase in specific activity. Vacuolar membranes were analyzed further by centrifugation through sucrose density gradients (3 h, 170,000 × *g*). ATPase activity formed a symmetrical peak at a density of 1.11 g/cm^3 (Fig. 1). Almost no vacuolar membrane ATPase activity was detected outside this peak, demonstrating that this enzyme is localized only on the vacuolar membrane. Profiles of activity for other vacuolar enzymes, α-mannosidase, AMPase, and protease, showed peaks coincident with ATPase activity. In contrast to ATPase, however, significant amounts of AMPase and α-mannosidase were also found toward the top of the gradient, where soluble proteins would occur.

The vacuolar membranes had apparently reached an equilibrium value at the low density of 1.11 g/cm^3 since a similar gradient profile of ATPase, AMPase, and α-mannosidase activities was obtained after centrifugation for 3 h at only 100,000 × *g* in another experiment (data not shown).

In an effort to increase the yield and specific activity of ATPase in vacuolar membrane preparations, several alternative lysis media were tested. In comparison with the usual medium of 1 mM EGTA (pH 7.5), 10 mM HEPES-NaOH (pH 7.5) resulted in similar recoveries of ATPase activity (units) but approximately twice as much protein. Surprisingly, the addition of 5 mM $MgCl_2$ to the HEPES-NaOH medium led to very poor recoveries of ATPase activity. In addition, unusually high levels of alkaline phosphatase activity were found in membranes after lysis in the HEPES-NaOH buffers (±$MgCl_2$). Although PMSF and BSA inhibited vacuolar membrane protease activity by 90%, the addition of PMSF (0.5 mM) or BSA (0.3%) to the EGTA wash medium resulted in somewhat lower (PMSF) or no different (BSA) yields of vacuolar membrane ATPase activity. Thus, EGTA proved to be the preferred lysis medium. Interestingly, repeated washing of vacuolar membranes in EGTA caused the loss of at least 80% of the ATPase activity.

Effect of inhibitors. To characterize the vacuolar membrane ATPase, we first examined the effects of several known ATPase inhibitors. In the experiment shown in Table 3, azide inhibited ATP hydrolysis by 11%. Our previous conclusion that azide acted by inhibiting contaminating mitochondrial ATPase was supported by the fact that oligomycin caused the same amount of inhibition.

Vanadate caused 22% inhibition in this experiment (Table 3). The effect of vanadate was analyzed more carefully by measuring vacuolar membrane ATPase activity over a range of vanadate concentrations, 0.05 to 500 μM (Fig. 2). For comparison, we also determined the effect of vanadate on ATPase activity of plasma membranes and on alkaline phosphatase activity of

TABLE 3. Effect of inhibitors on vacuolar membrane ATPase activity[a]

Addition	Sp act (μmol/min per mg)	Inhibition (%)
None	0.45	
Oligomycin	0.40	11
Azide	0.40	11
Vanadate	0.35	22
Azide, vanadate	0.31	31
Azide, vanadate, ethanol	0.34	
Azide, vanadate, DCCD	0.03	91

[a] The reaction mixtures contained 5 mM Na_2ATP, 5 mM $MgCl_2$, 10 mM NH_4Cl, and 10 mM PIPES, adjusted to pH 7.5 with Tris base. Additions included oligomycin (10 μg/ml), 5 mM KN_3, 20 μM Na_3VO_4, 0.1% ethanol, and 0.2 mM DCCD (diluted from a 0.2 M stock solution in 100% ethanol). See the text for details of ATPase activity assays in the absence or presence of DCCD.

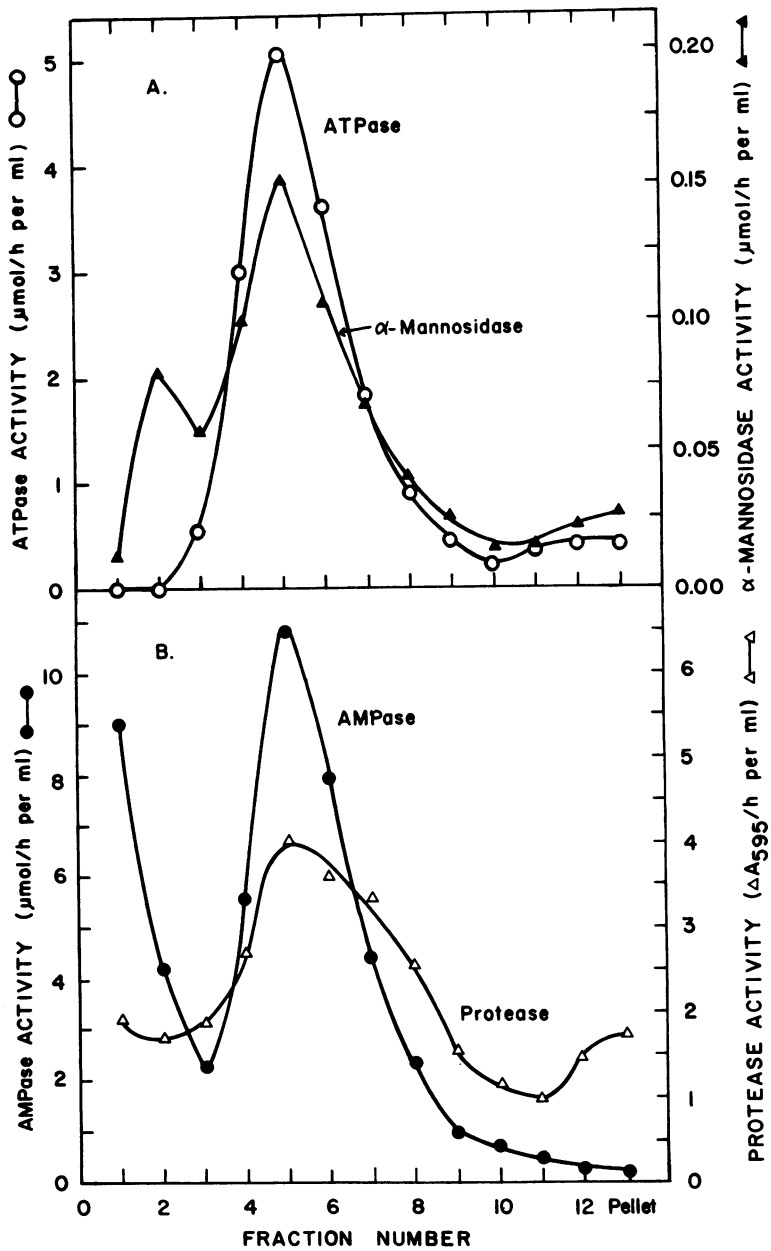

FIG. 1. Sucrose density gradient profile of vacuolar membrane fraction. Linear gradients containing 15 to 45% (wt/wt) sucrose were centrifuged, fractionated, and assayed for vacuolar membrane ATPase, α-mannosidase, AMPase, and protease activities as detailed in the text. Fraction 5 had a density of 1.11 g/cm^3.

vacuolar membranes. Both plasma membrane ATPase and vacuolar membrane alkaline phosphatase activities showed high sensitivity to vanadate, with K_i values of 1.0 and 10.0 μM, respectively. ATPase activity in vacuolar membranes was inhibited 26% by the highest vanadate concentrations, with half-maximal inhibition at approximately 10 μM, suggesting that the vanadate-sensitive portion of ATP hydrolysis was due to alkaline phosphatase and that the vanadate-insensitive ATP hydrolysis was due to the vacuolar membrane ATPase.

Thus, vacuolar membranes contain an ATPase that is not affected by specific inhibitors of either plasma membrane or mitochondrial ATPases. However, DCCD, a potent inhibitor of both of these proton-translocating ATPases (6), inhibited the vacuolar membrane ATPase by

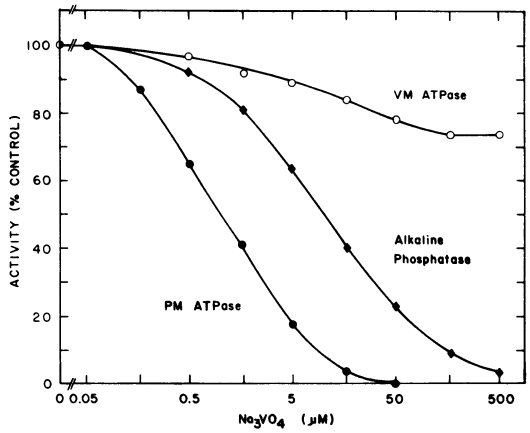

FIG. 2. Effect of vanadate on plasma membrane (PM) ATPase, vacuolar membrane (VM) ATPase, and vacuolar membrane alkaline phosphatase activities. Data for the plasma membrane ATPase were taken from Bowman et al. (5). Vacuolar membrane ATPase activity was assayed in medium containing 5 mM Na_2ATP, 5 mM $MgCl_2$, 10 mM NH_4Cl, 5 mM KN_3, and 10 mM PIPES, adjusted to pH 7.5, and various concentrations of Na_3VO_4 as shown. Assay medium for vacuolar membrane alkaline phosphatase activity contained 5 mM *p*-nitrophenylphosphate in place of ATP and was adjusted to pH 8.3. Enzymatic products, P_i or *p*-nitrophenol, were measured as described in the text. Control activities in the absence of Na_3VO_4 were 0.30 and 0.52 μmol/min per mg of protein for activities of vacuolar membrane ATPase and alkaline phosphatase, respectively.

91% (Table 3). Quantitative determination of ATPase activity as a function of DCCD concentration showed half-maximal inhibition at 2 μM and almost complete inhibition at 10 μM (Fig. 3). By contrast, alkaline phosphatase activity associated with vacuolar membranes was insensitive to inhibition by DCCD up to 30 μM (data not shown).

Substrate specificity. As a result of the inhibition studies, the vacuolar membrane ATPase could be most clearly defined as a DCCD-sensitive, azide- and vanadate-insensitive ATPase. We used these stringent criteria to examine the substrate specificity of the enzyme. The vacuolar membranes proved capable of hydrolyzing a number of phosphate compounds (Table 4). Among the trinucleotides, ATP was the best substrate, but GTP and ITP were hydrolyzed 75% as well, and UTP was hydrolyzed one-half as well as ATP. By contrast, CTP was hydrolyzed only slowly. DCCD (0.2 mM) inhibited hydrolysis of ATP, GTP, ITP, UTP, and CTP by 90 to 100%, and the amount of DCCD-sensitive activity found with each trinucleotide closely followed the substrate preference seen in the ethanol control.

Three substrates, ADP, phosphoenolpyruvate, and *p*-nitrophenylphosphate, were hydrolyzed only poorly by vacuolar membranes, with specific activities from 2 to 7% that of ATP hydrolysis in ethanol controls (Table 4). (Somewhat higher values were obtained in the absence of ethanol.) AMP, as shown previously (Table 1, Fig. 1), was hydrolyzed at relatively high rates, with a specific activity 121% that seen for ATP. However, AMPase clearly differed from ATPase in that AMPase was insensitive to DCCD (Table 4) and was completely active in the absence of $MgCl_2$ (data not shown).

In summary, the data in Table 4 show that the vacuolar membrane ATPase prefers ATP as a substrate but can also hydrolyze other trinucleotides (GTP, ITP, and UTP), at least in vitro. However, ATPase is apparently inactive on the other phosphate compounds examined.

Effect of divalent cations. The vacuolar membrane ATPase exhibited a strict requirement for a divalent metal cation (5 mM). There was no detectable activity in the absence of added salt (Table 5). Of the six cations tested, Mg^{2+} and Mn^{2+} were equally effective in stimulating ATPase activity. The activity with Co^{2+} was 47% that seen with Mg^{2+}; Fe^{2+}, Zn^{2+}, and Ca^{2+} were all less effective (26 to 29%).

Dependence upon ATP concentration. The rate of ATP hydrolysis was measured in reaction mixtures containing 5 mM $MgCl_2$ and ATP concentrations ranging from 0.05 to 5.0 mM. ATPase activity as a function of ATP concentration appeared to follow Michaelis-Menten kinetics with a K_m value for ATP of 0.2 mM (Fig. 4). Furthermore, no change in the K_m for sub-

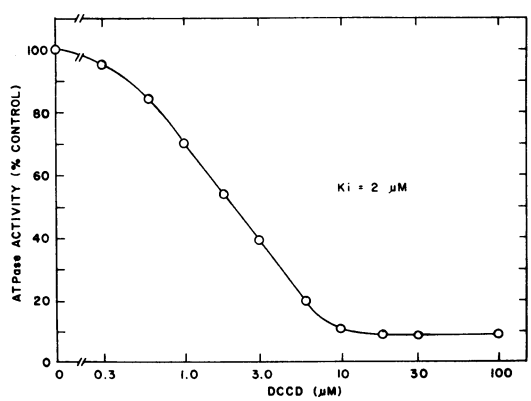

FIG. 3. Inhibition of vacuolar membrane ATPase activity by DCCD. Membranes were suspended in standard reaction mixtures with ATP omitted, DCCD was added to give the concentrations shown, and the mixtures were allowed to sit at 30°C for 5 min. The ATPase reaction was started by the addition of ATP. Control activity in the absence of DCCD was 0.44 μmol/min per mg of protein.

TABLE 4. Substrate specificity and effect of DCCD on substrate hydrolysis[a]

Substrate	Sp act				
	Ethanol control		+DCCD (μmol/min per mg)	DCCD-sensitive activity	
	μmol/min per mg	%[b]		μmol/min per mg	%[b]
ATP	0.42	100	0.00	0.42	100
GTP	0.34	81	0.03	0.31	74
ITP	0.32	76	0.00	0.32	76
UTP	0.21	50	0.00	0.21	50
CTP	0.04	10	0.00	0.04	10
ADP	0.02	5	0.01	0.01	2
AMP	0.51	121	0.50	0.01	2
Phosphoenolpyruvate	0.03	7	0.01	0.02	5
p-Nitrophenylphosphate	0.01	2	0.00	0.01	2

[a] The reaction mixtures contained 3 mM substrate, 5 mM $MgCl_2$, 10 mM NH_4Cl, 5 mM KN_3, 20 μM Na_3VO_4, and 10 mM PIPES, adjusted to pH 7.5 with Tris base. Activity (as P_i released) was assayed in the presence of either 0.1% ethanol or 0.2 mM DCCD as described in the text.

[b] Percentage of the activity seen with ATP.

strate was obtained when the substrate was assumed to be Mg · ATP and the Mg · ATP concentration was calculated by the Wolf-Adolph equation (50).

Effect of ions and buffers. The vacuolar membrane ATPase was remarkably indifferent to the presence and absence of a wide variety of ions. Among the monovalent cations (added as chloride salts), the enzyme showed a slight preference for NH_4Cl over KCl over NaCl (Table 6). In the presence of moderate concentrations (10 to 50 mM) of KCl, NaCl, and NH_4Cl, small stimulations of activity (26% maximal) were seen, whereas high concentrations (100 mM) resulted in small inhibitions (22% at most). These effects are considerably less than the three- to fivefold stimulation by KCl and NaCl of *Neurospora* mitochondrial ATPase (26) and even lower than the modest 30 to 70% stimulation by KCl and NH_4Cl of *Neurospora* plasma membrane ATPase (5, 8).

TABLE 5. Divalent cation requirement for ATPase activity[a]

Salt (5 mM)	Sp act	
	μmol/min per mg	%
No additions	0.00	0
$MgCl_2$	0.72	100
$MgSO_4$	0.85	118
$MnCl_2$	0.82	114
$CoCl_2$	0.34	47
$CaCl_2$	0.21	29
$ZnSO_4$	0.21	29
$FeCl_2$	0.19	26

[a] The reaction mixtures contained 5 mM Na_2ATP, 5 mM divalent cation salt, 10 mM NH_4Cl, 5 mM KN_3, 20 μM Na_3VO_4, and 10 mM PIPES, adjusted to pH 7.5 with Tris base.

The effects of a number of anions (as sodium or potassium salts) were tested. The specific activity of ATPase was inhibited by less than 20% by NaCl, $NaNO_2$, $NaHCO_3$, sodium acetate, Na_2SO_4, Na_2CO_3, and NaH_2PO_4 and was stimulated 16% by $(NH_4)_2SO_4$. (A similar stimulation by SO_4^{2-} was found with $MgSO_4$ in place of $MgCl_2$ in Table 5.) Only two anions had more pronounced effects on vacuolar membrane ATPase activity. At 50 mM, $NaNO_3$ and KSCN inhibited ATPase activity by 69 and 78%, respectively.

Comparison of ATPase activity at pH 7.5 with several buffer systems showed minor differences. The specific activity in the standard reaction mixture buffer, PIPES-Tris, was up to 20% higher than that in PIPES-NaOH, HEPES-Tris, or HEPES-NaOH buffer (all at 10 mM), no different from that in Tris-hydrochloride or Tris-acetate buffer (50 mM), and 20% lower than that in Tris-sulfate (50 mM) or β,β'-dimethylglutaric acid–Tris (20 mM) buffer.

DISCUSSION

Identification of vacuolar membrane ATPase. One hypothesis to explain the accumulation of basic amino acids into fungal vacuoles, supported by transport studies involving *S. cerevisiae*, is that vacuolar membranes contain a proton-translocating Mg^{2+}-ATPase which drives the accumulation of arginine and other basic amino acids (33). The results in this paper strongly suggest that such an ATPase is present in the vacuolar membranes of *N. crassa*. An ATPase activity was identified in membranes isolated from a vacuolar fraction which was purified 54-fold from the crude homogenate. The ATPase is strictly dependent on a divalent cation (Mg^{2+} or

TABLE 6. Effects of Na^+, K^+, and NH_4^+ on ATPase activity[a]

Addition (mM)	Sp act	
	μmol/min per mg	%
No additions	0.68	100
NaCl		
2	0.66	97
10	0.75	110
50	0.70	103
100	0.66	97
KCl		
2	0.72	106
10	0.74	109
50	0.80	118
100	0.56	82
NH_4Cl		
2	0.73	107
10	0.86	126
50	0.82	121
100	0.53	78

[a] The reaction mixture contained 5 mM Tris–ATP, 5 mM $MgCl_2$, 20 μM Na_3VO_4, oligomycin (10 μg/ml), and 10 mM PIPES, adjusted to pH 7.5 with Tris base.

Mn^{2+}) for activity and is strongly inhibited by DCCD, an inhibitor of proton translocation.

Comparison of the properties of this ATPase with those of the two well-studied proton-pumping ATPases of *Neurospora* plasma membranes and mitochondria shows that it is clearly distinct. The vacuolar membrane ATPase is largely resistant to inhibition by 20 μM vanadate, has a K_m for ATP of 0.2 mM, hydrolyzes GTP and ITP nearly as efficiently as ATP, and has a pH optimum of 7.5. By contrast, the plasma membrane ATPase is inhibited 95 to 100% by 20 μM vandate, has a K_m for ATP of 1.7 mM, is highly specific for ATP as a substrate, and has a pH optimum of 6.7 (5, 9). Although such major differences between vacuolar membrane and mitochondrial ATPases are not found, the vacuolar membrane ATPase is distinguishable from the mitochondrial ATPase by its insensitivity to the classical inhibitors of mitochondrial ATPase, KN_3 and oligomycin, and by a pH optimum of 7.5 rather than 8.3 (26).

The vacuolar ATPase appears to be tightly associated with the membrane. During sucrose density gradient centrifugation of the vacuolar membrane fraction, all of the ATPase activity remained associated with the membrane, showing a peak of activity at a density of 1.11 g/cm^3. Evidence that the material at a density of 1.11 g/cm^3 is indeed vacuolar membranes comes from the observation of comigration of the vacuolar marker enzymes α-mannosidase and protease with this peak. In addition, our results confirm a previous report (45) that some, but not all, of the α-mannosidase and protease activities are membrane associated. They also implicate AMPase activity as a third vacuolar marker enzyme which is partially membrane associated in *N. crassa*.

The observed density of 1.11 g/cm^3 in vacuolar membranes on sucrose gradients is considerably lower than the value of 1.17 g/cm^3 reported by Vaughn and Davis (45). The discrepancy can be attributed to the procedure for isolating membranes from vacuoles. Lysis and washing in 1 mM EGTA before gradient centrifugation probably removes many loosely associated proteins, thereby lowering the density of the membrane fraction. The low density of EGTA-washed vacuolar membranes has the advantage of contrasting sharply with the higher values of 1.15 and 1.18 g/cm^3 found with EGTA-washed plasma membranes and mitochondrial membranes, respectively (9; E. J. Bowman, unpublished data). Densities of approximately 1.11 g/cm^3 have also been found for isolated membranes of chromaffin granules and plant vacuoles (2, 4).

Properties and function of vacuolar membrane ATPase. Our data suggest that the vacuolar membrane ATPase belongs to the F_1F_0 family of ATPases, typified by the mitochondrial ATPase. Although distinguishable from the mitochondrial ATPase (see above), the vacuolar membrane ATPase closely resembles it in several characteristics. The vacuolar membrane and mitochondrial ATPases of *N. crassa* have almost identical substrate affinities for ATP (K_m values of 0.2 and 0.3 mM, respectively) and almost identical

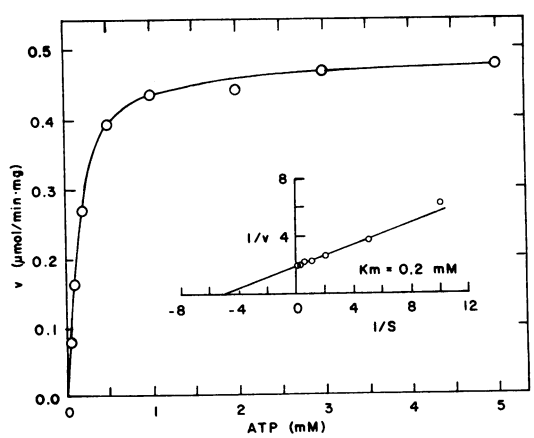

FIG. 4. Activity of vacuolar membrane ATPase as a function of substrate concentration. ATPase activity was determined in reaction mixtures containing 5 mM $MgCl_2$, 10 mM NH_4Cl, 5 mM KN_3, 20 μM Na_3VO_4, and 10 mM PIPES, adjusted to pH 7.5, and ATP to give the concentrations shown. A double-reciprocal plot of $1/v$ versus $1/S$ is shown in the inset.

substrate specificities in which ATP is preferred, but other trinucleotides (GTP and ITP) are cleaved at high rates (26). In addition, ATPase activity of vacuolar membranes, like that of mitochondrial membranes, is largely lost with repeated washings at 4°C in 1 mM EGTA, consistent with the existence of an easily removable, possibly cold-labile, F_1 complex (35).

These basic characteristics are shared by a number of other organellar ATPases, including those of chromaffin granules (1, 2, 23), lysosomes (39), and plant vacuoles (12). All differ from mitochondrial ATPases in their resistance to inhibition by oligomycin or azide but resemble mitochondrial ATPases in their substrate affinity, substrate specificity, divalent cation requirement, and DCCD sensitivity. Solubilization and purification of the proton-pumping ATPase from chromaffin granule membranes reveals an F_1F_0 ATPase structure where the F_1 sector is almost identical to mitochondrial F_1 ATPase as shown by molecular weight on gel filtration, subunit composition on denaturing gels, antigenic reactivity, and proteolytic fingerprints of the three largest subunits (3).

Even though direct evidence that the vacuolar membrane ATPase functions as a proton pump in *N. crassa* is lacking, several pieces of correlative evidence argue for such a function. First, as outlined above, of the two known types of eucaryotic ion pumps, the vacuolar membrane ATPase resembles the F_1F_0 ATPases, and all F_1F_0 ATPases appear to act as proton pumps (18). Second, the vacuolar membrane ATPase is highly sensitive to inhibition by DCCD, and DCCD inhibits other ATPases by binding to a specific subunit associated with proton movement (18, 42). Third, the lack of effect of a large number of salts on vacuolar membrane ATPase activity suggests that none of the ions tested plays an integral role in the enzyme mechanism. Fourth, vacuoles of *N. crassa* and *S. cerevisiae* are essentially identical organelles (45), and evidence that a proton motive force generated by an ATPase drives basic amino acid transport in vacuolar membrane vesicles of *S. cerevisiae* is available (33). Recently, Kakinuma et al. (21) have shown that the *S. cerevisiae* vacuolar membrane contains an ATPase with characteristics almost identical to those we have described for *N. crassa*.

In addition to driving transport, the *Neurospora* vacuolar membrane ATPase may well play other roles in vacuolar physiology. The suggestion has been made that vacuoles of both fungi and plants are essentially lysosomes because of their contents of phosphatases, nucleases, proteases, and lipases (4, 29–31, 49). *Neurospora* vacuoles, with their high content of basic amino acids and polyphosphates (11, 28, 45), also resemble chromaffin granules, which are characterized by high amounts of amines such as catecholamines and epinephrine and of phosphate compounds, namely, ATP (32). All of these organelles contain membrane ATPases that probably function as proton pumps involved in transport processes, pH maintenance, or both (21, 23, 27, 32, 33, 37, 40). Many questions concerning the mechanism of action of the ATPase and its physiological role in cellular metabolism remain. Because the physiological role of the vacuole in *N. crassa* is particularly well understood (13), further information on the biochemistry of the ATPase should readily lead us to an understanding of its function in vivo.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants GM-28703 from the National Institute of General Medical Sciences and RR-08132 from the Division of Research Resources, National Institutes of Health.

We are grateful to C. L. Cramer, R. H. Davis, T. J. Paulus, C. W. Slayman, and R. L. Weiss for helpful discussion.

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