Purification and Characterization of the Plasma Membrane ATPase of Neurospora crassa

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The plasma membrane of Neurospora crassa contains a proton-translocating ATPase, which functions to generate a large membrane potential and thereby to drive a variety of H⁺-dependent co-transport systems. We have purified this ATPase by a three-step procedure in which 1) loosely bound membrane proteins are removed by treatment with 0.1% deoxycholate; 2) the ATPase is solubilized with 0.6% deoxycholate in the presence of 45% glycerol; and 3) the solubilized enzyme is purified by centrifugation through a glycerol gradient. This procedure typically yields ~30% of the starting ATPase activity in a nearly homogeneous enzyme preparation of high specific activity, 61-98 µmol/min/mg of protein. The membrane-bound and purified forms of the ATPase are very similar with respect to kinetic properties (pH optimum, nucleotide and divalent cation specificity, sigmoid dependence upon Mg-ATP concentration) and sensitivity to inhibitors (including N,N-dicyclohexy lacarbodiimide and vanadate).

Upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the purified ATPase displays a single major polypeptide band of M₀ = 104,000, which is essentially identical in its electrophoretic mobility with the major subunit of [Na⁺, K⁺]-ATPase of animal cell membranes and [Ca²⁺]-ATPase of sarcoplasmic reticulum. The structural similarity of the fungal and animal cell ATPases, together with the fact that both are known to form acyl phosphate intermediates, suggests that they may share a common reaction mechanism.

There is now good evidence that the plasma membrane of the fungus Neurospora has, as its primary transport system, an ATP-dependent H⁺ pump which functions physiologically to generate a large membrane potential and to supply energy to a series of H⁺-dependent co-transport systems (1, 2). An important feature of this proton pump is that it lends itself to study by several different and mutually complementary techniques. For example, electrophysiological studies, made possible by the large diameter of Neurospora hyphae, gave the first indication of the existence of the pump (3, 4) and since then have provided useful information about its ATP dependence (5), mechanism (6, 7), and metabolic control (8). Current-voltage curves of the Neurospora membrane with and without inhibitors that block the pump have shown that the normal in vivo stoichiometry is 1 H⁺ transported/ATP hydrolyzed (6, 7) and that the major energetic transition during the reaction cycle occurs during the charge-transfer step (7). At the same time, with the use of pH microelectrodes, it has proven possible to study the role of the pump in the regulation of intracellular pH (9).

The first important step toward investigating the molecular mechanism of the pump came in 1977, when Scarborough (10) and we (11) showed that plasma membranes, isolated from the slime strain of Neurospora, possess an ATPase which is clearly the enzymatic equivalent of the ATP-dependent proton pump. This ATPase has a K₁/₂ for Mg-ATP of 1.8 mM (12), as predicted from whole cell studies (5), and it is insensitive to classical inhibitors of mitochondrial ATPase such as oligomycin and azide but sensitive to N,N-dicyclohexylcarbodiimide and vanadate (10-13).

Subsequent studies by Scarborough have focused on the way in which the ATPase functions in the plasma membrane. Using membrane vesicles from the slime strain, he has shown that: 1) ATP hydrolysis drives the uptake of [¹⁴C]SCN⁻, consistent with the idea that the vesicles are inverted, developing an inside-positive membrane potential (14); 2) at the same time, the interior of the vesicles becomes acidic, as evidenced by the uptake of [¹⁴C]imidazole and by the quenching of fluorescein-labeled dextran (15); and 3) during the hydrolysis of [³²P]ATP, a phosphorylated intermediate is formed (16).

As valuable as the vesicle studies have been, it is also important to purify the ATPase and determine its molecular properties. Studies of this kind can be expected to contribute information about the chemical nature of the ATPase reaction, and also to permit a closer comparison of the Neurospora enzyme with other energy-coupling ATPases. With these goals in mind, we set out to purify the plasma membrane ATPase of Neurospora. For the reasons outlined in the preceding paper (17), it seemed best to use membranes from the wild type strain, which contain high specific activities of the ATPase and very little contamination by other intracellular membranes. From these membranes, it has proved possible to solubilize the ATPase with detergent and to purify it to near-homogeneity by glycerol gradient centrifugation. The purified enzyme has been characterized with respect to kinetic properties and subunit composition and exhibits a remarkable structural similarity to membrane ATPases from animal cells. Preliminary descriptions of this work have been published (18, 19).

MATERIALS AND METHODS

Enzyme Purification—Plasma membranes, prepared as described in the preceding paper (17) and stored at −70°C, were quickly thawed and suspended at a protein concentration of 2 mg/ml in buffer containing 5 mM Na₂ATP, 2 mM EDTA, 0.2 mM KCl, 25 mM Tris, adjusted to pH 7.5. The suspension was stirred in an ice bath and deoxycholate was added to a final concentration of 0.1%. The mixture...
was then centrifuged at 100,000 × g (K,av) for 1 h. The pellet, which contained more than 80% of the plasma membrane ATPase activity, was resuspended at a protein concentration of 5 mg/ml in a solution containing 2 mM Na₂ATP, 2 mM EDTA, 0.3 M KCl, 45% glycerol (v/v), 25 mM Tris, pH 7.5. After the suspension was mixed with a Teflon/glass homogenizer, deoxycholate was added to a final concentration of 0.6%. This mixture was centrifuged at 100,000 × g for 1 h. The supernatant, containing 45 to 70% of the ATPase activity, was then layered on top of a linear gradient of glycerol (45 to 60%), dissolved in 2 mM Na₂ATP, 0.3% deoxycholate, 10 mM Tris, pH 7.5. Gradients were centrifuged at 170,000 × g for 26 h. Fractions were collected by puncturing the bottom of the tube and assayed for protein and ATPase activity. ATPase-containing fractions were routinely stored at −70 °C for further experiments. When a more concentrated solution of ATPase was required, gradient fractions were diluted with an equal volume of 1 mM ethylene glycol bis(N,N'-dicyclohexylcarbo- diimide; N,N,N',N'-tetramethylethlenediamine (0.015%) and ammonium persulfate (2 mg/ml). At the top of the gradient gel, a stacking gel was formed of 3% acrylamide, 0.07% bisacrylamide, 0.12 M Tris, pH 6.8. The running buffer was 0.2 M glycine, 0.05 M Tris, and 0.1% sodium dodecyl sulfate. Proteins to be loaded onto the gel were suspended in a solution of 2.0% sodium dodecyl sulfate, 1.0% β-mercaptoethanol, 0.12 M Tris, pH 6.8. The running buffer was 0.2 M glycine, 0.05 M Tris, and 0.1% sodium dodecyl sulfate. Proteins to be loaded onto the gel were suspended in a solution of 2.0% sodium dodecyl sulfate, 1.0% β-mercaptoethanol, 0.12 M Tris, pH 6.8. The running buffer was 0.2 M glycine, 0.05 M Tris, and 0.1% sodium dodecyl sulfate.

**METHODS**

**Materials**—Reagents—Deoxycholic acid, obtained from Sigma, was purified by the method of MacLennan (23) and kept as a molecular weight standards (P-galactosidase, phosphorylase a, bovine bean phospholipids) was obtained from Associated Concentrates. SDS-Polyacrylamide Gel Electrophoresis—Slab gels consisting of a linear gradient of increasing polyacrylamide concentration were formed from the following two solutions: 1) 8% acrylamide, 0.2% bisacrylamide, 20% urea, 0.37 M Tris, pH 8.8; and 2) 16% acrylamide, 0.4% bisacrylamide, 50% urea, 0.37 M Tris, pH 8.8. Polymerization was catalyzed by addition of N,N,N',N'-tetraacylamidodiether (0.015%) and ammonium persulfate (2 mg/ml). At the top of the gradient gel, a stacking gel was formed of 3% acrylamide, 0.07% bisacrylamide, 0.12 M Tris, pH 6.8. The running buffer was 0.2 M glycine, 0.05 M Tris, and 0.1% sodium dodecyl sulfate. Proteins to be loaded onto the gel were suspended in a solution of 2.0% sodium dodecyl sulfate, 1.0% β-mercaptoethanol, 0.12 M Tris, pH 6.8, and incubated for 30 min at 30 °C. The gels were stained and destained by the method of Fairbanks et al. (20).

**RESULTS**

**Purification**

**Removal of Loosely Bound Membrane Proteins**—Plasma membranes, isolated from wild type Neurospora by the method described in the preceding paper, have ATPase specific activities of 3 to 7 pmol/min/mg of protein. Early attempts at solubilizing the ATPase by relatively mild procedures such as repeated washes in 1 mM EDTA or 0.3 M KCl removed as much as 20% of the membrane protein but none of the ATPase activity. These results suggested that, unlike the F1 ATPase of mitochondria, chloroplasts, and bacteria, the integral membrane ATPase of Neurospora might be an integral membrane protein.

We therefore turned to detergents. Among several that were tried, deoxycholate gave the best preliminary results, especially when used in a two-step procedure modeled after the method of Kyte for purification of the [Na⁺, K⁺]-ATPase (24). The principle of the procedure was to use a low concentration of deoxycholate to remove loosely bound proteins, leaving a membrane fraction enriched in ATPase. The membranes were then disrupted with a higher concentration of deoxycholate, extracting the ATPase in a form that could be separated from other membrane components.

The details of the first step are illustrated in Fig. 1S (Miniprint). Plasma membranes were suspended in low concentrations of deoxycholate (from 0–0.3%), centrifuged at 100,000 × g for 1 h, and protein and ATPase activity were measured in the supernatant and pellet fractions. A range of deoxycholate concentrations was found (0.06–0.1%) at which 50–58% of the protein was removed but 85% or more of the ATPase activity remained in the pellet. Above this range, there was progressive inactivation of the ATPase; enzyme units in the pellet declined sharply and were not recovered in the supernatant.

**Solubilization of the ATPase in the Presence of Glycerol**—These results indicated that treatment with low deoxycholate concentrations was a useful first step, but suggested that detergent alone might be unable to remove the enzyme from the membrane in an active form. In a search for conditions that would promote solubilization with retention of activity, we took note of published reports that glycerol can be used to protect certain other membrane ATPases from inactivation by detergents (25, 26). Glycerol proved to be extremely effective with Neurospora as well. Fig. 2S illustrates the results of an experiment in which membranes, pretreated with 0.1% deoxycholate, were exposed to 0.6% deoxycholate in the presence of varying amounts of glycerol (0–60%, v/v) and then centrifuged at 130,000 × g for 1 h. About 35% of the membrane protein was solubilized by the deoxycholate, a value unaffected by the presence or absence of glycerol. Glycerol did, however, have a dramatic effect on the recovery of ATPase units. In the absence of glycerol, virtually all of the ATPase was inactivated, only a trace (2%) appearing in the pellet. By contrast, in the presence of 45% glycerol, there was excellent recovery of ATPase, of which a significant proportion (30% in the experiment of Fig. 2S) appeared in the supernatant.

In subsequent experiments, the glycerol concentration was fixed at 45% and protein and deoxycholate were varied in order to identify optimal concentrations. The best results were obtained with 0.6% deoxycholate and a membrane protein concentration of 5 mg/ml under these conditions, 60–70% of the ATPase appeared in the supernatant following centrifugation at 100,000 × g for 1 h.

**Purification on a Glycerol Gradient**—The solubilized ATPase could be separated from almost all other proteins by centrifugation through a linear gradient of glycerol (45–80%). In order for the ATPase to run as a symmetrical peak, it was necessary to include 0.3% deoxycholate in the gradient and to raise the deoxycholate concentration in the loaded sample to 1.0%. Fig. 3S illustrates the distribution of protein and ATPase activity in such a gradient which was centrifuged for 26 h at 170,000 × g; most of the protein remained at the top, while the ATPase peak moved to the center of the gradient. After centrifugation for 35 h (not shown), the ATPase had sedimented still further to the bottom of the tube, 70% appearing in the two lowest fractions and 30% in the pellet. In both cases, enzyme activity remained stable during glycerol gradient centrifugation.

Figs. 1S–7S are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 81M-1339, cite authors, and include a check or money order for $2.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
ient centrifugation, such that 80–100% of the enzyme units present in the initial load could be recovered by summing the individual gradient fractions.

**Effect of Phospholipids on ATPase Activity**—During the course of purification, the ATPase became increasingly dependent upon added phospholipids for maximal activity. Fig. 4S illustrates that, following glycerol-gradient centrifugation, ATPase activity was stimulated more than 2-fold by the inclusion of asolectin in the assay mixture. Optimal stimulation was seen at an asolectin concentration of 200 μg/ml. The same concentration of asolectin produced 10% stimulation of ATPase activity in the starting plasma membranes, 25% stimulation in the deoxycholate pellet, and 60% stimulation in the supernatant fraction following solubilization with 0.6% deoxycholate and glycerol (Table I). All calculations of recovery of enzyme activity have been based on assays in the presence of asolectin.

**Summary of the Purification Procedure**—The final purification procedure, based on the experiments just described, is diagrammed in Fig. 1, and the results of a representative purification are summarized in Table I. In this experiment, ATPase specific activity increased from 6.5 μmol/min/mg of protein in the starting plasma membranes to 13.0 μmol/min/mg of protein after treatment with 0.1% deoxycholate, 16.3 μmol/min/mg of protein following solubilization, and finally 96.6 μmol/min/mg of protein in the pooled fractions from the glycerol gradient. Although there was some loss of enzyme units at each step, the final recovery (27%) was very reasonable.

**Subunit Composition of the ATPase**—During the course of purification, protein samples were also analyzed by SDS-polyacrylamide gel electrophoresis, and the results are illustrated in Fig. 2. The ATPase from the peak fraction of the glycerol gradient (lane 5) possessed a single prominent polypeptide band of Mr = 104,000. Because *Neurospora* mycelium can contain troublesome amounts of protease activity, depending upon growth conditions, a control experiment was carried out.

**Table I**

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<th>Purification and yield</th>
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<td>ATPase activity was determined in the absence and presence of added phospholipids (PL), as described under &quot;Materials and Methods&quot;; yields were calculated on the basis of activity in the presence of phospholipids. Protein was determined by the method of Lowry et al. (22) except in the case of fractions from the glycerol gradient, where the dye-binding method of Bradford (22) was used. Two factors dictated this choice. 1) For the Bradford method but not the Lowry method, there was good agreement between the amount of protein applied to the gradient and the amount obtained by summing the individual fractions following centrifugation; and 2) subsequent experiments with purified <em>Neurospora</em> ATPase have shown that protein values obtained by the Bradford method agree well with values from direct amino acid analysis, whereas the Lowry method appears to overestimate protein by ~40%. In this experiment, use of the Lowry method for gradient fractions would have reduced the final specific activities to 36 μmol/min/mg of protein in the case of the pooled fractions, and 70 μmol/min/mg of protein in the case of the peak fraction.</td>
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<td>Specific activity</td>
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<td>Glycerol gradient peak fractions</td>
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*DOC, deoxycholate.*

in which 1 mM phenylmethanesulfonyl fluoride was added during plasma membrane isolation and enzyme purification. Phenylmethanesulfonyl fluoride did not cause any detectable change in the molecular weight of the final polypeptide but did lower the ATPase activity of the plasma membranes by 40%, so it was omitted in subsequent experiments.1

1 Recent work has suggested that the apparent lack of protease

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**Fig. 1. Flow chart for the purification of plasma membrane ATPase.** The composition of the buffer at each step is given under "Materials and Methods."
Strikingly, the $M_r = 104,000$ polypeptide of *Neurospora* was indistinguishable in its mobility from the large subunit of hog kidney [Na$^+$, K$^+$]-ATPase (lane 6). This finding prompted a careful search for a lower molecular weight glycoprotein in the *Neurospora* preparation that might correspond to the β subunit of [Na$^+$, K$^+$]-ATPase. However, staining of gels with periodic acid-Schiff reagent (20) did not give any indication of such a band. Staining of overloaded gels with Coomassie blue did, upon occasion, reveal minor polypeptide bands which were judged to be residual impurities rather than components of the ATPase: none of the minor bands was enriched relative to the starting material; none co-purified stoichiometrically with the $M_r = 104,000$ subunit; and the number and amount of the minor bands varied from one preparation to the next. The purity of the $M_r = 104,000$ polypeptide, measured by absorbance scanning, ranged from 70–90% in different preparations.

It was also possible, by absorbance scanning of gels, to estimate the enrichment of the $M_r = 104,000$ band during purification and to compare the relative enrichment of the purified ATPase specific activity. In the experiment of Table II, the $M_r = 104,000$ polypeptide comprised 6.2% of the total protein in the starting plasma membranes and increased to 12.2% of the low deoxycholate pellet, 35.8% of the high deoxycholate supernatant, and finally 70% of the peak fraction from the glycerol gradient. In this particular case, ATPase activity displayed roughly parallel increases (Table II). In other experiments, however, there has been as much as a 2-fold stimulation of total ATPase during the initial exposure to detergent, making it difficult to determine the quantitative relationship between the $M_r = 104,000$ polypeptide and enzyme activity.

**Properties of the Purified ATPase**

**Sensitivity to Inhibitors**—An identifying characteristic of the plasma membrane ATPase of *Neurospora* is its sensitivity to low concentrations of vanadate (12, 13). Fig. 5S shows that the purified ATPase retained this sensitivity and was inhibited by vanadate over the same range of concentrations as the membrane-bound enzyme, with $I_{50}$ values of 0.6 and 1.0 μM, respectively.

The purified ATPase also retained its sensitivity to DCCD, an inhibitor known to react with the proton channel of mitochondrial, chloroplast, and bacterial ATPases (27–29). The $I_{50}$ value for DCCD inhibition, calculated from the data in Fig. 6S, was 8 μM for both the membrane-bound and purified ATPase.

**pH Dependence**—The curve of activity as a function of pH is of particular interest for a proton-pumping ATPase because, in addition to the usual effects of pH on protein structure and activity, variations in pH alter the concentration of the transported ion. Fig. 7S shows that the pH dependence of the purified ATPase was essentially the same as that of the membrane-bound enzyme, with broad optima in both cases in the region of pH 6.5. This value is slightly more acidic than the cytoplasmic pH of *Neurospora*, recently measured with intracellular micropipette electrodes to be 7.1 (9).

**Substrate Specificity and Kinetic Behavior**—As shown in Table III, the purified ATPase closely resembled the membrane-bound ATPase in its specificity for nucleoside triphosphates and for divalent cations. Both forms of the enzyme were highly specific for ATP, giving little activity with other nucleoside triphosphates or with dATP, ADP, or AMP. Both membrane-bound and purified ATPases preferred Mg$^{2+}$ as divalent cation, but significant activity was also seen with Co$^{2+}$ and Mn$^{2+}$ (Table III).

A distinctive kinetic feature of the membrane-bound enzyme is the fact that it shows a sigmoid dependence of activity upon Mg$^{2+}$ concentration (12). This feature was retained by the purified ATPase, as shown in Fig. 3. The $K_s$ value for Mg-ATP was 1.8 mM (compared with 1.9 mM for the membrane-bound ATPase), and the Hill number, as a measure of cooperativity, was 1.8 (compared with 2.1; Ref. 12).

**Effect of Monovalent Cations**—There have been frequent suggestions that potassium influx may be linked directly to proton efflux in fungi (31–33) and in higher plants (34, 35). Our earlier work on the membrane-bound form of the *Neurospora* ATPase showed that there was indeed some stimulation of activity by NH$_4^+$ and K$^+$ and some inhibition by Na$^+$ (11, 12). However, detailed kinetic analysis gave no evidence for specific ion requirements of the sort exhibited by the [Na$^+$], [K$^+$], and [Mg$^{2+}$] dependent ATPase of *Neurospora*.
Plasma Membrane ATPase of Neurospora

**Fig. 3.** Activity of the purified ATPase as a function of substrate concentration. ATPase activity was assayed in the absence of monovalent cations as described in Ref. 13. The reaction mixture contained 10 mM PIPES buffer, adjusted to pH 6.7 with Tris base, and equimolar concentrations of MgCl₂ and Tris-ATP. Asolectin was added as described under “Materials and Methods.” The resulting ATPase activities have been plotted as a function of the added concentration of ATP and Mg²⁺. Values of [S]₀, determined from these data, were 1.9 mm for the membrane-bound ATPase (12) and 1.8 mm for the purified ATPase; and Hill numbers, determined from a plot of v/(V_max - v) versus S, were 2.1 and 1.5, respectively. When the equation of Wolf and Adolph (30) was used to calculate the actual concentrations of [Mg-ATP] complex present in the various reaction mixtures, the values of [S]₀ decreased to 1.3 and 1.2 mM, but the Hill numbers (1.7 and 1.6) still indicated cooperativity.

**Fig. 4.** Effect of monovalent cations on the activity of the purified ATPase. The reaction mixture contained 5 mM MgCl₂, 5 mM Tris-ATP, 10 mM PIPES buffer adjusted to pH 6.7 with Tris base, and varying concentrations of NH₄Cl, KCl, or NaCl. Asolectin was added as described under “Materials and Methods.” Control ATPase activity in the absence of added monovalent cations was 39 μmol/min/mg of protein.

K⁺-ATPase of animal cells or the [H⁺,K⁺]-ATPase of gastric mucosa and instead suggested that changes in the activity of the Neurospora enzyme were simple salt effects, unrelated to transport function (12). Fig. 4 shows that NH₄⁺, K⁺, and Na⁺ had similar effects on the purified form of the Neurospora ATPase. The enzyme, assayed in 10 mM Tris-PIPES buffer, had very high activity (39 μmol/min/mg of protein) in the complete absence of added monovalent cations. It could be stimulated 2-fold by the addition of fairly high concentrations of NH₄Cl (50 mM) or KCl (50 mM). NaCl (50 mM) increased activity by 40%, representing a small difference between the purified enzyme and the membrane-bound enzyme. There was no synergistic effect of Na⁺ and K⁺. For example, adding 10 mM NaCl plus 10 mM KCl stimulated activity by 65%, a value intermediate between the values determined for each salt individually. In the presence of 125 mM NaCl plus 10 mM KCl, (a typical assay mixture for the [Na⁺,K⁺]-ATPase), activity was stimulated by 34%.

**DISCUSSION**

**Purification Method**—The procedure described in this paper for the purification of the Neurospora plasma membrane ATPase gives an enzyme preparation which is 70-90% pure, has high specific activity, and can be obtained in good yield. The failure of milder treatments to release the ATPase from the membrane and the requirement for a relatively high concentration of deoxycholate indicate that the enzyme is an integral membrane protein, a conclusion strengthened by the fact that it needs added phospholipid for maximal activity. However, detergents alone seem incapable of solubilizing the ATPase in an active form, and it was the use of glycerol which finally made possible the successful purification outlined above. Although glycerol appears essential, there is greater latitude in the choice of detergent. Cholate and octaethyl-neglycoldodecyl ether (C₁₂E₈), in conjunction with glycerol, are both capable of solubilizing the ATPase with little loss of activity.²

**Properties of the Purified Enzyme**—In repeated preparations, the purified ATPase, as collected from the glycerol gradient, has had specific activities ranging from 61 to 98 μmol/min/mg of protein. The apparent co-enrichment of ATPase activity and the Mᵢ = 104,000 polypeptide during the experiment of Table II suggests that, in the best preparations, there is very little inactivation of the enzyme. Quantitative data on this point are difficult to obtain, however, because of sporadic detergent stimulation of the ATPase³ and because of the assumptions involved in calculating protein content from densitometric scans of Coomassie-stained gels. In any event, once purified, the ATPase is quite stable. It has retained more than 95% of its activity during 6 months of storage at −70 °C, and even after 3 months of storage at 4 °C, 68% of the original activity was still present.³

² B. J. Bowman, unpublished experiments.

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The purified ATPase resembles very closely the membrane-bound form of the enzyme with respect to pH optimum, nucleotide and divalent cation specificity, cooperative kinetics, and sensitivity to inhibitors. The fact that it retains sensitivity to DCCD may be of interest in view of the known interaction of this inhibitor with the proton channel of mitochondrial, chloroplast, and bacterial ATPases (27–29). Further experiments will be required to identify the DCCD-binding site (or sites) of the Neurospora plasma membrane ATPase.

Subunit Composition—One of the most striking features of the purified ATPase is the fact that, upon SDS-polyacrylamide gel electrophoresis, it contains a single major polypeptide band of M, = 104,000. Thus, it falls structurally into the class of transport ATPases that includes the [Na+,K+]-ATPase of animal cells (36), the [Ca++]-ATPase of sarcoplasmic reticulum (37), and the [Hi,K+]-ATPase of gastric mucosa (38). In fact, in the gel system that was employed in the present study, the Neurospora plasma membrane ATPase was identical in its electrophoretic mobility with the α subunit of hog kidney [Na+,K+]-ATPase; a previous study showed it also to be essentially identical with [Ca++]-ATPase of sarcoplasmic reticulum (19). For this entire group of ATPases, there is still active debate as to whether SDS-polyacrylamide gel electrophoresis provides an accurate measure of molecular weight (36, 39). Therefore, until the work described in this paper is extended by SDS-polyacrylamide gels run over a range of acrylamide concentrations (36), gel filtration in the presence of detergent (39), and/or equilibrium centrifugation (37), the molecular weight calculated here for the subunit of the Neurospora plasma membrane ATPase must be regarded as an approximation. The important point for present purposes is the close resemblance between the Neurospora ATPase and the three transport ATPases from animal cells.

Very similar results have been reported recently for two other fungal ATPases. Using lysolecithin as detergent, Goffeau and co-workers solubilized and purified the plasma membrane ATPase from the fission yeast Schizosaccharomyces pombe and found it to contain a single polypeptide band of M, = 100,000 (40) to 103,000 (41). Likewise, by means of the zwitterionic detergent 3-(tetradecyldimethylammonium)-1-propanesulphate, Malpartida and Serrano (42) achieved partial purification of the corresponding ATPase from Saccharomyces cerevisiae, and found a polypeptide band of M, = 105,000 to be greatly enriched in their preparation. And finally, upon incubating plasma membranes from the slime strain of Neurospora with [γ-32P]PjATP, Dame and Scarborough (16) observed a phosphorylated band at M, = 105,000 that appeared, based on several criteria, to correspond to the plasma membrane ATPase.

Besides the remarkable similarity in subunit composition, the fungal and animal ATPases share other important structural and kinetic features. All of them form acyl phosphate intermediates during the course of the reaction cycle (16, 23, 41, 43, 44); and all are sensitive to inhibition by vanadate (12, 45), which is believed to act as a transition state analog of phosphate (44). Furthermore, in the Neurospora plasma membrane ATPase (12) and in all of the animal ATPases (43, 44, 46), there is evidence for two ATP binding sites which interact with one another cooperatively. Thus, in spite of the fact that these ATPases have been firmly established to transport different cations, further work may reveal that they possess a common enzymatic mechanism.

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**Figure 18:** Removal of protein from plasma membranes by treatment with low concentrations of deoxycholate. Plasma membranes were suspended at a density of 200 mg/ml in 20 mM Tris-HCl, pH 7.5, and were treated with 0.1% deoxycholate for 1 h. The protein concentration was 0.25 mg/ml.

**Figure 19:** ATPase activity of protein and supernatant fractions of plasma membranes. The ATPase activity was determined as described in Materials and Methods. The specific activity of the control was 2.7 μmol/min/mg protein. There was no detectable ATPase activity in the supernatant fractions.

**Figure 20:** Solubilization of plasma membrane ATPase activity. Plasma membranes, which had been previously washed in 0.14 M NaCl as described in Materials and Methods, were suspended in a protein concentration of 3.5 mg/ml in a solution containing 2 mM MgCl2, 3 mM KCl, 0.2 M NaCl, 25 mM Tris adjusted to pH 7.5, and varying concentrations of glycerol. The supernatants were then dialyzed against a 0.5 M KCl solution containing 0.1 M glycerol, and the ATPase activity was determined as described in Materials and Methods. The ATPase activity was inhibited by 0.5 M KCl and ATPase activity was determined as described in Materials and Methods.

**Figure 21:** Specific activity of ATPase in the supernatant was 1.5 μmol/min/mg protein. The specific activity of the control was 2.7 μmol/min/mg protein. There was no detectable ATPase activity in the supernatant fractions.

**Figure 22:** Effect of pH on ATPase activity. Activity was assayed as described in ref. 13, except that the pH was varied by titration of 10 mM HEPPS with Tris base, and amiloride was present in the case of the purified ATPase (see Materials and Methods).