Solubilization and Purification of the Neurospora Plasma Membrane H\(^+\)-ATPase*

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The electrogenic proton-translocating ATPase in the plasma membrane of Neurospora has been solubilized with lysolecithin and purified using a combination of gel filtration and density gradient centrifugation. Isolated plasma membrane vesicles are solubilized with lysolecithin in the presence of MgATP, vanadate, and chymostatin. The MgATP and vanadate are required to maintain the ATPase in an active form and the chymostatin prevents proteolytic degradation. Optimal solubilization of the H\(^+\)-ATPase (approximately 70%) occurs at a lysolecithin/protein ratio of 3 (mg/mg). The lysolecithin extract is then passed through a Sepharose CL-6B column in the presence of lysolecithin, deoxycholate, MgATP, vanadate, dithiothreitol, and chymostatin. The bulk of the H\(^+\)-ATPase activity elutes from this column as a relatively broad peak near the void volume. Sodium dodecyl sulfate-polyacrylamide gel analysis of the column fractions indicates that the H\(^+\)-ATPase present in the first half of this peak is substantially separated from all other membrane proteins whereas the H\(^+\)-ATPase in the second half of the peak is still significantly contaminated. Isopycnic glycerol density gradient centrifugation of the pooled fractions from each half of the column peak results in further purification of the H\(^+\)-ATPase. Gradient fractions containing the H\(^+\)-ATPase from the first half of the column peak are virtually free of any other membrane proteins and those from the second half of the column peak are only slightly contaminated. The overall yield of the H\(^+\)-ATPase is at least 24% based upon enzyme activity, and the specific activity is approximately 10 \(\mu\)mol of Pi liberated/mg of protein/min.

In previous reports from this laboratory, we have described a method for isolating plasma membranes from the eukaryotic microorganism, Neurospora, in high yield and purity (1, 2), characterized the biochemical properties of the ATPase which exists in the isolated plasma membranes (3), directly demonstrated that the ATPase catalyzes electrogenic (4) proton translocation (5), identified the hydrolytic moiety of the H\(^+\)-ATPase as an approximately 105,000-dalton protein and demonstrated that its catalytic mechanism involves a phosphoryl-enzyme intermediate (6), and identified the phosphorylated amino acid as \(\beta\)-aspartyl phosphate (7). This enzyme has also been studied in the laboratory of Slayman (8–10), and several laboratories have reported studies of a similar ATPase in yeast (11–21), although electrogenic proton translocation, kinetic competence of the phosphoryl-enzyme intermediate, and the nature of the phosphorylated amino acid have not yet been demonstrated for the yeast enzyme.

On the basis of all of these studies, it has become apparent that the Neurospora plasma membrane H\(^+\)-ATPase, and probably the yeast ATPase as well, bears a striking resemblance to the cationmotive Na\(^+/K\(^+\)- and Ca\(^{2+}\)-translocating ATPases of animal cell origin (22, 23). The marked similarities between these three enzymes suggest that they may all operate via the same general mechanism, and accordingly, we have recently proposed a common mechanistic model (7) based upon the ligand conduction concepts of Mitchell (24).

A major goal in this laboratory is to detail the precise molecular events which transpire when the Neurospora plasma membrane H\(^+\)-ATPase transduces the chemical energy of ATP hydrolysis into a transmembrane electrochemical proton gradient. An important prerequisite for progress toward this goal is delineation of the subunit composition of the functional proton pump, i.e., to clarify whether or not any protein(s) other than the hydrolytic moiety are involved in the proton-translocating mechanism. The resolution-reconstitution approach pioneered for membrane proteins primarily in the laboratory of Racker is the most powerful approach available for answering such questions, and in order to use this approach for determining the subunit composition of the Neurospora H\(^+\)-ATPase, a method for purifying this enzyme in a catalytically active form was needed.

In this communication, we report a method for solubilizing and purifying the hydrolytic moiety of the Neurospora H\(^+\)-ATPase in active form essentially free of other proteinaceous plasma membrane constituents. The method involves the use of the detergent lysolecithin and represents an adaptation of the method of Dufour and Gouffe (12) which successfully exploited this detergent for solubilizing and purifying the plasma membrane ATPase of Schizosaccharomyces pombe.

**EXPERIMENTAL PROCEDURES**

**Growth of Cells and Isolation of Plasma Membrane Vesicles**

Cells of the Neurospora crassa sl strain were grown and plasma membrane vesicles isolated as previously described (2) except that the "resuspension buffer" was 0.01 M 2-(N-morpholino)ethanesulfonic acid (pH 6.8 with Tris) and \(\alpha\)-methylmannoside recrystallized from hot (85 °C) water was used. In addition, chymostatin (1 \(\mu\)g/ml) was added to all solutions used in the plasma membrane isolation procedure after the cell lysis step.

**Solubilization of the H\(^+\)-ATPase**

The solubilization buffer (20 ml) is prepared as follows. To 18 ml of H\(_2\)O are added 0.1 ml of 0.2 M ATP/MgSO\(_4\) (pH 6.8 with Tris) and

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0.2 ml of 0.01 M NaVO₄ (unbuffered and at least 1-month-old), and the pH adjusted to 7.5 with MES¹ or Tris. The volume is then brought to 20 ml with H₂O and lysosolcin (20 mg) and chymostatin (2 µl of a 20 µg/ml solution in dimethylsulfide) are added. If the resulting mixture is cloudy, it is sonicated to clarity by sonication if necessary) at a ratio of 3 ml/g of vessel protein and the mixture is allowed to come to room temperature (approximately 24 °C) gradually over a period of 15 min. The extract is then centrifuged (183,000 × g, 45 min, 10 °C) and the resulting supernatant fluid is concentrated to one-half of its original volume by filtration through an Amicon CF₂₅ cone (10 °C, 1200 × g for approximately 10 min for 3 ml of solution). If the volume is reduced to less than one-half the original volume, the concentrate is made back up to this volume using the filtrate. This is the preparation referred to hereafter as the solubilized extract. At this point, aliquots of the solubilized extract are removed for determinations of the ATPase activity and protein content.

**Purification of the H⁺-ATPase**

**Step I: Chromatography on Sepharose CL-6B**—Approximately 8 ml of the solubilized extract (2 to 3 mg of protein) is further concentrated in an Amicon CF₂₅ cone to a volume of about 2 ml and immediately applied to the bottom of a Sepharose CL-6B column (1.5 × 30 cm, 1.0 litre, 7 ml of gradient containing the bulk of the ATPase activity). Aliquots of the solubilized extract are removed for determinations referred to hereafter as the solubilized extract. At this point, portions (7 ml) from the Sepharose CL-6B column are layered on top of a glycerol density gradient fractionator and assayed for ATPase activity. The bulk of the ATPase activity emerges from the column in a broad peak (approximately 18 ml) which begins near the column volume. In the experiment described in Tables I and II, fractions representing the leading and trailing halves of this peak were pooled and designated pooled early fractions and pooled late fractions, respectively.

**Step II. Glycerol Density Gradient Centrifugation—Pooled fractions (7 ml) from the Sepharose CL-6B column are layered on top of a 30 ml linear glycerol density gradients (20 to 40% glycerol w/v) in a solution containing ATP (2 mM), EDTA (2 mM), chymostatin (2 µg/ml), dithiothreitol (1 mM), lystosolcin (1 mg/ml), deoxycholate (1 mg/ml), and dithiothreitol (1 mM), adjusted to pH 7.5 with Tris. The volume is eluted with this solution and after 25 ml have passed through the column, 1-ml fractions are collected and assayed for ATPase activity. The bulk of the ATPase activity emerges from the column in a broad peak (approximately 18 ml) which begins near the column volume. In the experiment described in Table I, fractions representing the leading and trailing halves of this peak were pooled and designated pooled early fractions and pooled late fractions, respectively.

**Standard ATPase Assay**

Incubation mixtures contained 5 to 10 µl of the various ATPase preparations, 5 µl of 200 mM ATP/MgSO₄ (pH 6.8 with Tris), 5 µl of 100 mM NaN₃ (pH 6.8 with MES), 40 µl of 100 mM MES (pH 6.8 with Tris), and 10 µl of Folch Fraction I suspension (5 mg/ml in 0.1 M MES, pH 6.8 with Tris), in a reaction volume of 100 µl. In all assays except those of the glycerol gradient fractions, in addition to these components, the incubations contained 10 µM NaVO₄ (added with the aliquot of ATPase to be assayed, or pre-added in certain cases) and 2 mM EDTA (pH 6.8 with NaOH) which reverses the vanadate inhibition. Reactions were initiated by the addition of enzyme, allowed to proceed at 30 °C for 10 to 20 min, and terminated by the addition of 0.1 ml of 5% (w/v) sodium dodecyl sulfate solution (25). The inorganic phosphate content in the entire 0.2-ml samples was then measured as described by Stanpton (26). Reagent blanks were obtained by adding the SDS before the ATPase preparations.

¹The abbreviations used are: MES, 2-(N-morpholino)ethanesulfonic acid; NaVO₄, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; danseyl, 5-dimethylaminonaphthalene-1-sulfonyl.

**Materials**

Bovine serum albumin, MES, ATP (Tris salt low in vanadate), α-methylmannoside, Folch Fraction I (type I), deoxycholate (free acid), and lysosolcin were from Sigma. Acrylamide, N,N'-methylenebisacrylamide, and sodium dodecyl sulfate were from Gallard-Schleising. Tris, glycine, and SDS-PAGE molecular weight standards were from Bio-Rad. Sodium orthovanadate, analyzed as described by Stroobant and Scarborough (31), was from Fisher. Phosphatidylserine, phosphatidylcholine, phosphatidylinositol, phosphatidylglycerol, and phosphatidylethanolamine were from Miles. Asolectin was from Associated Concentrates, Woodside, NY. Other reagents were of the purest commercially available grade.

**RESULTS**

**Proteolytic Degradation of the H⁺-ATPase: The “Nicking” Phenomenon—Our routine procedure for preparing Neurospora plasma membrane vesicles involves isolation of large quantities of concanavalin A-stabilized plasma membrane ghosts, storing the isolated ghosts at -70 °C in 90% glycerol, and preparing vesicles from the stored ghosts as needed, by treatment with α-methylmannoside (2). When it became possible to identify the Neurospora plasma membrane H⁺-ATPase in SDS-polyacrylamide gels (6), a proteolytic degradation problem became apparent. Freshly prepared vesicles usually contained the hydrolytic moiety of the ATPase predominantly as an approximately 105,000 dalton species, but during storage, the ATPase became degraded, in stepwise fashion, to 100,000, 90,000, and 80,000 dalton species. Furthermore, even freshly prepared vesicles were sometimes found to contain partially degraded ATPase. In order to gain control of this nicking phenomenon, we screened a large variety of commercially available protease inhibitors and found that chymostatin, a peptide elaborated by certain strains of Streptomyces (32), very effectively prevents the nicking phenomenon during storage. Furthermore, when this protease inhibitor is added to the solutions used for
preparation of the membranes, nicking during the isolation procedure is also largely prevented. The ATPase nicking phenomenon can be seen in Fig. 1 which shows a stained SDS-polyacrylamide gel of vesicles prepared from ghosts isolated and stored in the presence of chymostatin (lane 1) and vesicles prepared from ghosts isolated and stored in the absence of this protease inhibitor (lane 2). This figure also shows the relative mobility of the (Na+/K+)ATPase purified from hog kidney, for reasons that will be discussed below. Subsequent experiments indicated that nicking is also a problem during solubilization and purification of the ATPase. However, chymostatin also effectively prevents degradation of the ATPase during these procedures, and accordingly, is included in all of the experiments described below.

Solubilization of the H+-ATPase—Table I describes certain of the conditions required for optimum solubilization of the Neurospora H+-ATPase by lysolecithin. As was found by Dufour and Goffau with the yeast plasma membrane ATPase (12), the presence of ATP in the solubilization mixture is important for obtaining efficient solubilization of the ATPase. The inclusion of MgSO₄ or vanadate with the ATP does not significantly improve upon the extraction carried out with ATP alone, but the inclusion of MgSO₄ plus vanadate reproducibly augments the amount of ATPase activity solubilized. Vanadate alone is ineffective. Interestingly, unlike the results of Dufour and Goffau, EDTA is detrimental in the extraction procedure, and this negative effect is greater than what would be predicted solely on the basis of its ability to form a complex with Mg²⁺. The essential point to be made from the data presented in Table I is that optimum solubilization of the ATPase requires the presence of ATP, MgSO₄, and vanadate.

![Fig. 1. The ATPase nicking phenomenon.](image)

The ATPase nicking phenomenon. Plasma membrane ghosts were isolated and stored at -70 °C for 3 months, in the presence or absence of chymostatin. At that time, vesicles were prepared from each of the stored ghost preparations in the presence of MgATP and vanadate. Each entry represents the extraction of 1 mg of vesicle protein. Where appropriate, vanadate was added to the individual ATPase assay mixtures so that the final concentration of Na₃VO₄ in each assay mixture was 10 μM. The reported values are the average of at least three experiments.

<table>
<thead>
<tr>
<th>Additions to basal solubilization medium</th>
<th>Per cent vesicle ATPase activity solubilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM ATP</td>
<td>40.5</td>
</tr>
<tr>
<td>1 mM ATP + 1 mM MgSO₄</td>
<td>38.4</td>
</tr>
<tr>
<td>1 mM ATP + 100 μM Na₃VO₄</td>
<td>40.8</td>
</tr>
<tr>
<td>1 mM ATP + 1 mM MgSO₄ + 100 μM Na₃VO₄</td>
<td>62.9</td>
</tr>
<tr>
<td>100 μM Na₃VO₄</td>
<td>17.1</td>
</tr>
<tr>
<td>1 mM ATP + 1 mM MgSO₄ + 1 mM EDTA</td>
<td>9.8</td>
</tr>
</tbody>
</table>

![Fig. 2.](image)

The effect of increasing concentrations of vanadate on the per cent solubilization of protein and H+-ATPase activity from the Neurospora plasma membrane vesicles. Extractions were carried out as described under “Experimental Procedures” except that the concentration of Na₃VO₄ was varied. Each point represents the extraction of 1 mg of vesicle protein. Where appropriate, vanadate was added to the individual ATPase assay mixtures so that the final concentration of Na₃VO₄ in each assay was 10 μM.

The effect of vanadate on the efficiency of ATPase solubilization is concentration-dependent as is shown in Fig. 2, where solubilization of the ATPase in the presence of MgATP was measured as a function of the concentration of vanadate in the solubilization mixture. It can be seen that while the amount of protein solubilized is independent of the concentration of vanadate, the amount of ATPase activity solubilized increases as a function of the vanadate concentration, reaching a maximum around 100 μM. Importantly, SDS-PAGE analysis of the solubilized material in a similar experiment (not shown) demonstrated that the total amount of the hydrolytic moiety solubilized is independent of the vanadate concentration, indicating the vanadate increases the solubilization of active ATPase rather than ATPase per se. The implications of these findings will be discussed below.

The relative amounts of lysolecithin and membrane protein present in the solubilization mixture are important for optimal solubilization of active ATPase as is shown in Fig. 3, where the yield of solubilized ATPase activity was measured as a function of the ratio of lysolecithin to membrane protein (mg/mg). The greatest yield of active ATPase (about 70% on the average) is obtained at a ratio of 3, where about half of the
membrane protein as a function of the lysolecithin/protein ratio. Extractions were carried out as described under "Experimental Procedures" except that the ratio of lysolecithin to membrane protein was varied by varying the volume of the solubilization buffer. Each point represents the extraction of 1 mg of vesicle protein. The solubilized extracts were reduced to one-half their volume by filtration through an Amicon CF25 cone and 19 µl of each was assayed for ATPase activity.

membrane protein is solubilized. At higher ratios, there is a decrease in the yield of the ATPase which is probably due to inhibition of the ATPase activity by higher concentrations of lysolecithin rather than a decrease in the amount solubilized. Quantitative densitometry of Coomassie blue-stained gels of the vesicles and lysolecithin-extracted vesicles also demonstrated approximately 70% solubilization of the ATPase at a ratio of 3 which indicates that lysolecithin is not significantly inhibitory at a ratio of 3 or below.

The lysolecithin-solubilized ATPase requires exogenously added lipid for maximal activity. Numerous lipids and detergents have been assessed for their ability to stimulate the lysolecithin-solubilized ATPase, including phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylglycerol, phosphatidylserine, Asolectin, Folch fraction I, Lubrol PX, and Tween 20, 40, and 80 (data not shown). Of these, Folch fraction I, a crude lipid fraction from bovine brain containing predominantly phosphatidylinositides, phosphatidylserine, and cerebroside, proved to be the best, and is thus a component of our standard assay system.

Attempts to improve further upon the solubilization conditions thus far described, including alteration of the MgATP concentration, sonication, the addition of various salts or glycerol, varying the pH of the solubilization mixture, and varying the solubilization time and temperature, did not result in any improvement in the yield of active ATPase. Thus, on the basis of these experiments, our standard procedure for solubilizing the ATPase involves treatment of the vesicles with lysolecithin at a ratio of 3 mg/mg of membrane protein in the presence of 1 mM MgATP, 0.1 mM vanadate, and 2 µg/ml of chymostatin, at pH 7.5 for 15 min during which time the temperature increases from 0–24 °C. The H⁺-ATPase solubilized in this manner loses only about 25% of its activity when stored at 4 °C for 2 days and is totally stable for at least a week at –20 °C.

Purification of the H⁺-ATPase—The various steps in the ATPase purification procedure are described in Table II and Fig. 4. Table II lists the conventional parameters reported in enzyme purification schemes and Fig. 4 shows Coomassie blue-stained SDS-PAGE analysis of the membrane proteins

![Fig. 3. Solubilization of H⁺-ATPase activity and plasma membrane protein as a function of the lysolecithin/protein ratio. Extractions were carried out as described under "Experimental Procedures" except that the ratio of lysolecithin to membrane protein was varied by varying the volume of the solubilization buffer. Each point represents the extraction of 1 mg of vesicle protein. The solubilized extracts were reduced to one-half their volume by filtration through an Amicon CF25 cone and 19 µl of each was assayed for ATPase activity.](image)

![Fig. 4. SDS-PAGE analysis of the Neurospora plasma membrane H⁺-ATPase at the various stages of purification. Lane I, molecular weight standards: myosin, β-galactosidase, phosphorylase B, bovine serum albumin, and ovalbumin with Mr = 200,000, 116,116, 97,114, 66,296, and 42,807, respectively (36). Lane 2, Neurospora plasma membrane vesicles, 50 µg of protein. The upper arrow indicates the hydrolytic moiety of the H⁺-ATPase, Mr ~ 105,000; the lower arrow points to the doublet with Mr ~ 55,000 and 53,000. Lane 3, solubilized extract, 30 µg of protein. Lanes 4 and 5 are the pooled early fractions and pooled late fractions from the Sepharose CL-6B column, respectively (15 and 30 µg of protein). Lanes 6 and 7 are the glycerol gradient-purified, pooled early and late Sepharose CL-6B column fractions (approximately 2.6 and 1.4 µg of proteins, respectively).](image)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Specific Activity</th>
<th>Activity Recovery</th>
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</thead>
<tbody>
<tr>
<td>Vesicles</td>
<td>3.16</td>
<td>6.00</td>
<td>0.95</td>
<td>5.70</td>
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<tr>
<td>Solubilized extract</td>
<td>8.90</td>
<td>2.76</td>
<td>1.29</td>
<td>3.56</td>
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<tr>
<td>Sepharose CL-6B</td>
<td></td>
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<tr>
<td>Pooled early fractions</td>
<td>9.00</td>
<td>0.369</td>
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<td>Pooled late fractions</td>
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<td>1.84</td>
<td>1.07</td>
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<tr>
<td>Glycerol gradient of pooled early fractions</td>
<td>4.00</td>
<td>0.064</td>
<td>11.11</td>
<td>0.711</td>
</tr>
<tr>
<td>Glycerol gradient of pooled late fractions</td>
<td>4.00</td>
<td>0.068</td>
<td>9.42</td>
<td>0.641</td>
</tr>
</tbody>
</table>

*Not corrected for losses due to sampling for gel analyses, ATPase assays, and protein determinations.*

This value represents the average specific activity of the ATPase in the two most active gradient fractions. It was calculated from two separate determinations of the ATPase activity and protein content of these fractions.

*The small differences in the distance of migration of the ATPase in the individual lanes are due to the fact that the relative mobility of the ATPase in this SDS-PAGE system increases slightly as the amount of ATPase in a given sample increases (unpublished observations). The differences are not due to nicking; smaller amounts of the glycerol gradient-purified ATPase electrophoresed with a mobility essentially identical with the ATPase in lanes 3 and 5 (not shown). The reasons for this curious phenomenon are as yet unknown.*
The ATPase purification procedure per se results in some purification of the ATPase, which is evidenced by the increase in ATPase specific activity from 0.95 pmol of ATP hydrolyzed/mg of protein/min in the vesicles to 1.29 in the solubilized extract. This is largely due to the fact that the extraction procedure extracts vary little of one major membrane protein (M, ~ 50,000) as can be seen by comparing lanes 2 and 3 in Fig. 4.

The first major step in the purification scheme is chromatography of the solubilized extract on Sepharose CL-6B in the presence of MgATP, vanadate, lysolecithin, deoxycholate, dithiothreitol, and chymostatin, which are all necessary for the best results. Under these conditions, the majority of the ATPase (approximately 80%) emerges from the column as a broad peak of activity near the void volume (not shown) indicating that it is present as large aggregates. The ATPase molecules which emerge first (presumably the larger aggregates) are very effectively separated from most of the other membrane proteins, whereas those that emerge later are significantly contaminated. This can be seen in the SDS-PAGE profiles of the pooled early and late ATPase fractions presented in Fig. 4 (lanes 4 and 5) and is corroborated by the difference in ATPase specific activity in the pooled early and late fractions (3.2 versus 1.84, respectively) as indicated in Table II. After approximately 80% of the ATPase activity applied to the column is recovered, the remaining fractions are discarded because their specific activity is less than that of the starting material. As will be seen below, the ATPase can be separated from many of the membrane proteins by sedimentation through a glycerol density gradient. However, a protein doublet in the 54,000-dalton molecular mass range (lower arrow in Fig. 4) migrates very near the H+-ATPase in glycerol density gradients and is therefore difficult to remove by gradient sedimentation techniques. The advantage of the Sepharose CL-6B column step is that it removes this doublet entirely from the ATPase present in the early fractions and significantly diminishes it in the late fractions.

The final ATPase purification step involves sedimentation of the ATPase present in the Sepharose CL-6B column fractions through a linear 20 to 40% (w/v) glycerol density gradient containing lysolecithin, deoxycholate, ATP, EDTA, and chymostatin. During this step, virtually all of the remaining membrane proteins are removed from the ATPase contained in the early column fractions and nearly all are removed from the ATPase contained in the late column fractions. Lanes 6 and 7 of Fig. 4 indicate the purity of the gradient-purified, pooled early and late fractions, respectively. The only bands apparent in lane 6 are the ATPase, small amounts of nicked ATPase, and some low molecular weight material at and near the dye front. Part of the low molecular weight material is an electrophoretic artifact since lanes with no sample stain significantly at the dye front in this SDS-PAGE system. The rest is concanavalin A which arises from the plasma membrane isolation procedure. This conclusion was reached on the basis of an experiment in which the purified ATPase was analyzed on an exponential gradient SDS-polyacrylamide gel which clearly resolves concanavalin A into three bands. The only bands present in this gel (not shown) were the ATPase, small amounts of the nicked ATPase, and small amounts of three bands which ran in positions identical with the three bands in the concanavalin A standard. Thus, the only plasma membrane proteins present in the gradient-purified ATPase preparation obtained from the Sepharose CL-6B column fractions are the ATPase and small amounts of the nicked ATPase. The gradient-purified ATPase obtained from the late column fractions is only slightly more contaminated (lane 7), and the purity of this fraction can be improved on a second glycerol gradient (22 to 32% glycerol (w/v), not shown). Neither of the final ATPase preparations contains detectable amounts of glycoprotein, as judged by dansylhydrazine staining (28) of SDS-gels of these preparations (not shown).

The behavior of the ATPase in the gradient centrifugation step can be seen in Fig. 5 which shows the results of the gradient purification of the pooled late fractions obtained from the Sepharose CL-6B column. Most of the contaminating membrane proteins remain at the top of the gradient while the ATPase enters the gradient to a significant extent. This behavior is similar to that reported by Dufour and Goffeo for the yeast plasma membrane ATPase (17) which suggests that the aggregate molecular weight of the Neurospora plasma membrane H+-ATPase in lysolecithin is approximately one million.

The recovery of the ATPase activity from each of the gradients is about 90% of the activity applied, and the final specific activities of the ATPase from the glycerol density gradients of the pooled early and late fractions from the Sepharose CL-6B column are 11.11 and 9.42, respectively. These specific activities are quite similar to those now being reported for the plasma membrane ATPase of S. pombe (16). The overall final recoveries of the ATPase reported in Table II are lower than the actual recoveries because these data are not corrected for the amounts of the enzyme preparations taken for gel analyses and determinations of the ATPase activity and protein content during the various steps of the purification.

At present, we do not consider the minor contamination by concanavalin A to be a serious problem, because the major reason for developing this purification procedure was to set the stage for delineating the subunit structure of the H+-ATPase by reconstitution methods, and the presence of small amounts of concanavalin A should not hinder these studies.

Notes:
1 The presence of three protein bands in commercial sources of concanavalin A has been discussed by Wang et al. (33).
isoatation procedure. If such corrections are made, the yield approximately doubles. The procedure can be carried out in about a day and a half, and the gradient-purified ATPase is fully stable for at least 5 weeks at -20 °C.

**DISCUSSION**

To summarize the experiments described in this communication, methods have been developed for solubilizing the hydrolytic moiety of the electronegative, proton-translocating ATPase from the plasma membrane of *Neurospora* and purifying it to near homogeneity. The H'-ATPase is extracted from the plasma membranes with lyssolecithin in the presence of MgATP, vanadate, and chymostatin. The MgATP and vanadate are required to maintain the ATPase in an active form, and the chymostatin prevents proteolytic degradation. The lyssolecithin extract is then passed through a Sepharose CL-6B column which affects a significant purification, and most importantly, substantially removes a protein doublet which is difficult to separate from the ATPase in glycerol density gradients. The column-purified ATPase is then further purified by isopycnic glycerol density gradient centrifugation. The only detectable plasma membrane proteins present in the most highly purified ATPase preparation are the hydrolytic moiety and small amounts of a nicked form of the hydrolytic moiety.

Ever since the existence of the *Neurospora* plasma membrane ATPase was demonstrated in this laboratory (1), we have been attempting to develop methods for solubilizing and purifying it in an active form. However, until recently, all attempts to do this were uniformly unsuccessful. Progress toward this end came as a result of two important developments. The first was the identification of the hydrolytic moiety of the ATPase in polyacrylamide gels (6), and the second was the report by Dufour and Goffeau (12) which described the use of lyssolecithin for solubilizing the plasma membrane ATPase of yeast. When this detergent was tried with the *Neurospora* H'-ATPase, encouraging results were immediately obtained, and a systematic investigation of several variables in the extraction procedure led to a procedure which reproducibly resulted in the solubilization of about 30-40% of the ATPase activity. However, the ability to identify the ATPase in SDS-polyacrylamide gels brought to light a significant problem with this extraction procedure, i.e. that the hydrolytic moiety was partially degraded (i.e. nicked) by membrane-associated protease activity when the membranes were solubilized with lyssolecithin. When the nicking problem was solved by inclusion of the protease inhibitor, chymostatin, in the solubilization mixture, the yield of active ATPase in the extraction procedure was improved somewhat, but comparison of the yield of active ATPase to the amount of hydrolytic moiety that was actually extracted (as judged by quantitative densitometry of stained SDS-polyacrylamide gels) indicated that a significant proportion of the ATPase was extracted in an intact but inactive form. Earlier experiments on the differential sensitivity of the ATPase to tryptic cleavage in the presence and absence of MgATP (3) had suggested that the ATPase may exist in different conformational states, and we have recently reported that the ATPase inhibitor, vanadate, augments the protective effect of MgATP against tryptic inactivation (6). Thus, with the idea that in the presence of MgATP and vanadate, the ATPase might be trapped in a state more conducive to extraction in active form, the effect of vanadate in the extraction procedure was investigated. The results (described in Table I and Fig. 2) indicated that vanadate (in the presence of MgATP) significantly enhances the solubilization of active ATPase molecules. This result is of obvious practical value, and moreover, provides additional evidence that the ATPase undergoes significant conformational changes during its catalytic cycle.

After developing optimal conditions for solubilizing the ATPase in an active form, again guided by the protocol of Dufour and Goffeau (12), attempts to purify the enzyme by glycerol density gradient centrifugation methods were initiated. The results indicated that while most of the lyssolecithin-solubilized membrane proteins could be separated from the hydrolytic moiety of the ATPase on such gradients, a doublet of approximately 53,000 to 55,000 daltons similar to that seen by Malpartida and Serrano in *Saccharomyces cerevisiae* plasma membranes (19) reproducibly co-purified with the ATPase. However, it was subsequently found that chromatography of the lyssolecithin extract on Sepharose CL-6B effectively separates this doublet from the bulk of the ATPase activity. Thus, chromatography on Sepharose CL-6B and glycerol density gradient sedimentation are both required for obtaining highly purified H'-ATPase from *Neurospora* plasma membranes.

Around the time that the lyssolecithin procedure for solubilizing and purifying the *Neurospora* plasma membrane H'-ATPase was being developed in this laboratory, a monograph describing a deoxycholate method for solubilizing and purifying this enzyme was published (34), and recently, a second monograph on the same subject has appeared (35). The specific activities of the purified ATPase in these reports were 25 and 39, respectively, which would appear to be significantly higher than those reported here (approximately 10). Some of this apparent discrepancy can be explained on the basis of differences in the ATPase assay conditions. In the aforementioned reports, the ATPase was assayed in the presence of 11 mM (NH₄)₂SO₄. The purified ATPase described herein is stimulated approximately 1.8-fold by this concentration of (NH₄)₂SO₄ (not shown) which would raise the specific activity to near 20. However, we prefer not to include NH₄⁺ in our standard assay because this ion abolishes ATP-dependent ΔH₂ generation in the isolated vesicles. Another factor which may be involved in the apparent discrepancy relates to the ATPase nicking phenomenon shown in Fig. 1. As described in a recent report from this laboratory, a nicked form of the H'-ATPase can be experimentally produced by treatment of the membranes with trypsin in the presence of MgATP plus vanadate (6), and in similar experiments we have recently found that a related nicked form (Mr = 98,000) is approximately 2.5-fold more active than the degraded ATPase. In one of the abovementioned monographs (35), the purified *Neurospora* ATPase was shown to co-migrate in SDS-polyacrylamide gels with the large subunit of the (Na⁺/K⁺)ATPase from hog kidney. In our hands, it is a nicked form of the *Neurospora* ATPase that co-migrates with the large subunit of the hog kidney ATPase (see Fig. 1), which suggests the possibility that the ATPase described in the aforementioned reports was partially degraded and more active for this reason. The difference in the strains of *Neurospora* used for the ATPase isolation is another potential factor that may be involved in the discrepancy. The ATPase preparation with a specific activity of 39 was apparently obtained from a wild type strain of *Neurospora* whereas the studies described herein were carried out with the cell wall-less *S. cerevisiae* strain. Conceivably, the wild type ATPase is inherently more active than the ATPase in the plasma membrane of the *S. cerevisiae* cells. Thus, the combined effects of (NH₄)₂SO₄, nicking, and strain differences can probably account for the difference in the specific activities of the purified ATPase observed in the two laboratories.

What remains at this level of our investigation of the

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*Unpublished results.*
molecular structure and mechanism of the Neurospora plasma membrane H⁺-ATPase is the development of a method for reconstituting the proton-translocating capability of the ATPase into artificial lipid vesicles so that the participation of any subunits other than the hydrolytic moiety can be confirmed or denied. The results described in this communication should facilitate the achievement of this important task. Determination of the subunit composition of the H⁺-ATPase would bring us one step closer to an understanding of the molecular mechanism of this enzyme, and in view of the marked similarities of the cationmotive ATPases of animal cells, it can be anticipated that what is learned about the mechanism of the Neurospora H⁺-ATPase should contribute to our understanding of the molecular mechanism of ion-translocating ATPases in general.

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