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Identification of the Hydrolytic Moiety of the *Neurospora* Plasma Membrane H^+-ATPase and Demonstration of a Phosphoryl-Enzyme Intermediate in Its Catalytic Mechanism†

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ABSTRACT: The hydrolytic moiety of the electrogenic, proton-translocating ATPase (ATP phosphohydrolase, EC 3.6.1.3) in the plasma membrane of *Neurospora* has been identified in Coomassie blue stained sodium dodecyl sulfate–polyacrylamide slab gels on the basis of its differential susceptibility to tryptic cleavage in the presence or absence of MgATP. Treatment of isolated *Neurospora* plasma membrane vesicles with trypsin in the absence of MgATP leads to a marked (~85%) inhibition of the ATPase activity whereas the enzyme activity is essentially unaffected when the membranes are treated with trypsin in the presence of MgATP. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of control membranes, membranes treated with trypsin, and membranes treated with trypsin in the presence of MgATP allows the identification of a single protein (M_r ~105 000) that responds differentially to trypsin in the presence or absence of MgATP. Trypsin treatment of the membranes in the absence of MgATP causes the disappearance of the 105 000-dalton protein whereas trypsin treatment in the presence of MgATP results in the removal of only a small polypeptide (~4000 daltons). Incubation of the membranes with [γ-^{32}P]ATP under appropriate conditions, followed by acidic sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography of the dried gel, allows the delineation of one major phosphorylated protein. This protein displays differential susceptibility to tryptic cleavage in the presence or absence of MgATP and is thus identified as the 105 000-dalton protein. Importantly, quantitative isotope exchange experiments indicate that essentially all the ATP hydrolysis catalyzed by these membranes proceeds via phosphorylation and dephosphorylation of the 105 000-dalton protein. These results demonstrate that the 105 000-dalton protein is the hydrolytic moiety of the electrogenic, proton-translocating ATPase and that the mechanism of ATP hydrolysis catalyzed by this enzyme involves a covalent phosphoryl-enzyme intermediate. The possible implications of these findings for the mechanisms of ion-translocating ATPases in general are discussed.

In the preceding paper (Scarborough, 1980), the accumulation of experimental evidence concerning the physiological role of the ATPase located in the plasma membrane of *Neurospora* is reviewed. In summary, electrophysiological studies with intact cells (Slayman, 1970; Slayman et al., 1973) and biochemical studies with isolated plasma membrane vesicles (Scarborough, 1976, 1980) clearly indicate that the *Neurospora* plasma membrane ATPase is an electrogenic proton pump.

A major objective in this laboratory is an understanding of the molecular mechanism by which the *Neurospora* plasma membrane ATPase transduces the chemical energy of ATP hydrolysis into a transmembrane electrochemical proton gradient. Many potential experimental approaches to this end, such as immunochemical analysis of the orientation of the enzyme in the membrane, identification of the catalytic and ionophoric sites on the enzyme, delineation of possible subunit structure, isolation–reconstitution, and studies of the partial reactions in the catalytic sequence, require prior identification of the ATPase molecule. The most conventional approach to

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identifying any enzyme is to solubilize and purify it, and some success with this approach has been reported for the *Neurospora* (Bowman et al., 1978a) and yeast (Dufour & Goffeau, 1978) plasma membrane ATPases. However, there are potential pitfalls inherent in this approach. The plasma membrane of *Neurospora* appears to contain several minor ATPase activities of unknown function (unpublished experiments) in addition to the electrogenic, proton-translocating ATPase. Therefore, unless the yield during purification is kept high, the identity of an isolated ATPase could be questionable. Furthermore, cells of *Neurospora* are known to contain at least 25 different proteolytic enzymes (Spady & Gaertner, 1978), and even the purified plasma membrane vesicles are not totally free of protease activity (unpublished experiments). Thus, without full attention to the potential problem of proteolytic cleavage during purification, it is possible to purify a proteolytic artifact.

In view of these potential difficulties, it was considered desirable to first identify the ATPase in the isolated plasma membrane vesicles, where it is known to be functional (Scarborough, 1976, 1980; Stroobant & Scarborough, 1979b), before attempting to solubilize and purify it. In the studies presented here, we identify the hydrolytic moiety of the *Neurospora* plasma membrane ATPase in sodium dodecyl sulfate–polyacrylamide gels on the basis of its differential susceptibility to tryptic cleavage in the presence or absence of Mg–ATP. The identification is confirmed by the demonstration that this protein is phosphorylated and dephosphorylated at a rate that is consistent with the overall rate of ATP hydrolysis catalyzed by the membranes. These findings establish the identity of the hydrolytic moiety of the *Neurospora* plasma membrane ATPase and, in addition, demonstrate the participation of a phosphoryl-enzyme intermediate in the catalytic mechanism of this proton pump.

Experimental Procedures

Isolation of Neurospora Plasma Membrane Vesicles. Plasma membrane vesicles were isolated as described by Stroobant & Scarborough (1979a) except that the "resuspension buffer" was 0.01 M MES[1] (pH 6.8 with Tris), and in the experiment described in Figure 3, methyl α-mannoside recrystallized from hot (85 °C) water was used.

ATPase Assays. Assays conducted at 30 °C contained 5 μL of 0.2 M ATP–$MgSO_4$ (pH 6.8 with Tris), 70 μL of 0.01 M MES (pH 6.8 with Tris), 5 μL of 0.1 M NaN_3 [to inhibit traces of mitochondrial ATPase activity (Bowman et al., 1978b)], and 20 μL of membrane vesicle suspension [1.3–2.9 mg of protein/mL in 0.01 M MES (pH 6.8 with Tris)]. The reactions were started by the addition of membranes, allowed to proceed for 5 or 10 min, and stopped by the addition of 0.4 mL of ice-cold 0.63 M perchloric acid. The resulting membrane suspensions were then mixed and centrifuged (1900*g*, 10 min), and the inorganic phosphate in the supernatant fluids was estimated essentially by the method of Stanton (1968). Assays conducted at 0 °C contained 32.5 μL of 0.2 M ATP–$MgSO_4$ (pH 6.8 with Tris), 32.5 μL of 0.1 M NaN_3, 65 μL of H_2O or 65 μL of 2 mM Na_3VO_4 (pH 6.8 with MES), and 520 μL of membrane vesicle suspension [3.7 mg of protein/mL in 0.01 M MES (pH 6.8 with Tris)]. The reactions were started by the addition of membranes, and at 10-min intervals, duplicate 100-μL aliquots were removed and mixed with 0.4 mL of ice-cold 0.63 M perchloric acid. The resulting membrane suspensions were then treated as described above for the 30 °C ATPase assays. Interference by vanadate in the inorganic phosphate determination was not significant in this experiment.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. High-resolution analytical $NaDodSO_4$–polyacrylamide slab gel electrophoresis was performed by using the gel system described by Laemmli (1970) except that the resolving gels contained 0.2% $NaDodSO_4$. The stacking and resolving gels contained 5 and 7.5% acrylamide, respectively. In both gels, the acrylamide/*N*,*N*′-methylenebis(acrylamide) ratio was 30:0.8. The membranes were disaggregated at 100 °C for 5 min in 0.05 M Tris (pH 6.8 with HCl) containing 1% $NaDodSO_4$, 10% glycerol, and 1% β-mercaptoethanol. Bromophenol blue was used as the tracking dye. Electrophoresis was conducted at 20–25 °C at constant current (50 mA, gel size 0.15 × 14 × 10 cm) for 2.5 h in a Bio-Rad Model 220 gel electrophoresis apparatus modified so as to allow temperature regulation of the lower electrophoresis buffer.

For acidic gels, the pH 2.4 $NaDodSO_4$–polyacrylamide gel electrophoresis system of Fairbanks & Avruch (1972) was used with the following modifications. A two-phase slab gel was prepared which included a stacking gel containing 4% acrylamide [40:1.5 acrylamide/*N*,*N*′-methylenebis(acrylamide)], 1% $NaDodSO_4$, and 0.05 M H_3PO_4 (pH 2.4 with NaOH) and a resolving gel containing either 5.6 or 7.5% acrylamide [40:1.5 acrylamide/*N*,*N*′-methylenebis(acrylamide)], 1% $NaDodSO_4$, and 0.25 M H_3PO_4 (pH 2.4 with NaOH). The final concentrations of the ascorbic acid, $FeSO_4 \cdot 7H_2O$, and H_2O_2 were increased to 110, 2.8, and 27 μg/mL, respectively. The membranes were disaggregated in 0.05 M H_3PO_4 (pH 2.4 with NaOH) containing 1% $NaDodSO_4$ for 15 min at room temperature. Aliquots were removed for protein assay, and the remainder of the sample was diluted 1:1 (v/v) with 0.05 M H_3PO_4 (pH 2.4 with NaOH) containing 0.5 M sucrose, 1% $NaDodSO_4$, and 0.02 mg/mL pyronin *Y*. Electrophoresis was performed at 20 °C at constant current (110 mA, gel size 0.15 × 14 × 10 cm) for 4.5–5 h in the Bio-Rad apparatus described above.

Gel Analyses. After electrophoresis, gels were either stained overnight at room temperature in 5:1:5 (v/v/v) methanol–acetic acid–water containing 0.25% Coomassie brilliant blue R250 or dried immediately with a Bio-Rad gel dryer. Stained gels were destained at room temperature, first in 500 mL of the staining solvent without dye for 2–3 h with one change and then in 500 mL of 1:1:18 (v/v/v) methanol–acetic acid–water for 5–18 h with several changes. Gels containing ^{32}P-labeled membrane proteins were stained and destained in less than 24 h. Autoradiography of gels containing ^{32}P-labeled membrane proteins was performed by exposing Kodak RP-X-O-Mat X-ray film to the dried gels. The exposure time was reduced by using a Du Pont Cronex Lightning Plus X-ray intensifying screen and conducting the autoradiography at −70 °C. Exposure times are indicated in the appropriate figure legends.

Stained gel and autoradiogram densities were scanned with a Joyce–Loebl densitometer. The resulting scans were integrated by excising and weighing three copies of the appropriate peaks.

Other. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Radioactivity was determined by liquid scintillation counting in Scintiverse or Cerenkov counting in H_2O.

Materials. MES, Tris, bovine serum albumin, ATP (Tris salt, low in vanadate), and methyl α-mannoside were obtained from Sigma. Acrylamide, *N*,*N*′-methylenebis(acrylamide),

[1] Abbreviations used: MES, 2-(*N*-morpholino)ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate.

Table I: ATP Hydrolysis Catalyzed by Control Membranes, Membranes Treated with Trypsin, and Membranes Treated with Trypsin in the Presence of MgATP[a]

membranes	ATP hydrolysis (nmol of P_i released mg^{-1} of protein min^{-1}) expt 1	expt 2
(a) control	879	795
(b) trypsin treated	174	86
(c) trypsin treated in the presence of MgATP	894	841

[a] To 1.23-mL aliquots of a plasma membrane vesicle suspension [1.72 mg of protein (experiment 1) and 1.60 mg of protein (experiment 2)] in 0.01 M MES and 0.244 mM Na_3VO_4[2] (pH 6.8 with Tris) were made the following additions: (a) 0.27 mL of 0.01 M MES (pH 6.8 with Tris); (b) 0.15 mL of 0.01 M MES (pH 6.8 with Tris) and 0.12 mL of trypsin solution (1 mg/mL in H_2O); (c) 0.15 mL of 0.2 M ATP-$MgSO_4$ (pH 6.8 with Tris) followed 30 s later by 0.12 mL of trypsin solution. After 2 min at 22 °C, reaction a was terminated by the simultaneous addition of 0.6 mL of soybean trypsin inhibitor solution (1 mg/mL in H_2O) and 0.12 mL of trypsin solution, and then the reaction was placed on ice. Reactions b and c were terminated 2 min after the addition of trypsin by the addition of 0.6 mL of soybean trypsin inhibitor solution, and then the reactions were placed on ice. The membranes were pelleted by centrifugation (12000*g*, 5 min), resuspended in 3 mL of ice-cold 0.01 M MES (pH 6.8 with Tris), and pelleted again by centrifugation. The membranes were then resuspended in ice-cold 0.01 M MES (pH 6.8 with Tris) (experiment 1, 0.45 mL; experiment 2, 0.90 mL) and assayed for protein content and ATPase activity (30 °C) as described under Experimental Procedures.

sodium dodecyl sulfate, ammonium persulfate, and Coomassie brilliant blue R250 were obtained from Bio-Rad. Sodium orthovanadate [analyzed as described by Stroobant & Scarborough (1979b)], NaN_3, and Scintiverse were from Fisher. TPCK-trypsin, soybean trypsin inhibitor, β-galactosidase, phosphorylase *a*, and human serum albumin were from Worthington. [γ-^{32}P]ATP (triethylammonium salt; specific activity 2–10 Ci/mmol or 1000–3000 Ci/mmol)[3] was obtained from New England Nuclear, and [α-^{32}P]ATP (triethylammonium salt) and [^{32}P]H_3PO_4 were obtained from Amersham. All other chemicals were of reagent grade or of the highest purity obtainable from commercial sources.

Results

Differential Trypsin Sensitivity of the Neurospora Plasma Membrane ATPase. ATP hydrolysis catalyzed by the *Neurospora* plasma membrane ATPase is markedly inhibited by treatment of the membranes with trypsin; however, in the presence of MgATP, the enzyme activity is unaffected by this proteolytic enzyme (Scarborough, 1977). We call this phenomenon differential trypsin sensitivity. Table I shows the results of two experiments in which ATPase activity was measured in control membranes and membranes treated with trypsin in the presence or absence of MgATP. It can be seen that exposure of the membranes to trypsin results in a marked inhibition of the ATPase activity (80% and 89%) whereas in the presence of MgATP the ATPase activity is completely protected against inhibition by trypsin. The most reasonable interpretation of these results is that one or more peptide bonds in the ATPase molecule are exposed and susceptible to tryptic cleavage in the absence of MgATP and that cleavage of these

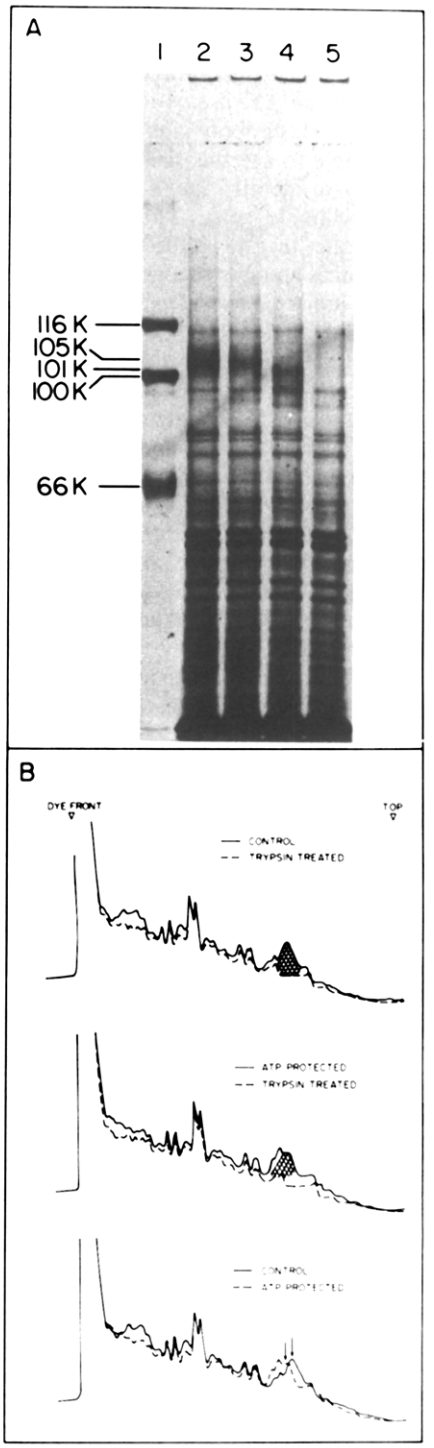

FIGURE 1: Identification of the ATPase on the basis of differential trypsin sensitivity. (A) Untreated plasma membrane vesicles and plasma membrane vesicles from experiment I in Table I were disaggregated and electrophoresed with the modified Laemmli system as described under Experimental Procedures (40 μg of protein/well). The resulting gel was then stained and destained as described under Experimental Procedures. Well 1, β-galactosidase, phosphorylase *a*, human serum albumin; well 2, untreated vesicles; well 3, control vesicles; well 4, vesicles treated with trypsin in the presence of MgATP; well 5, trypsin-treated vesicles. (B) Comparative densitometer scans of wells 3, 4, and 5 from (A). The ATPase is indicated by the cross-hatching. The slight shift in molecular weight of the ATPase in ATP-protected vesicles is indicated by the arrows in the third set of traces.

[2] Vanadate, a potent inhibitor of the *Neurospora* plasma membrane ATPase (Bowman & Slayman, 1979), enhances the ability of MgATP to protect the ATPase against inhibition by trypsin (unpublished experiments).

[3] Some batches of the high specific activity [γ-^{32}P]ATP were not suitable for use in these studies due to the presence of an unknown radioactive contaminant that caused an extremely high background in the autoradiograms; the lower specific activity [γ-^{32}P]ATP is preferable.

bonds results in the loss of ATP hydrolytic activity. In the presence of MgATP, however, these critical linkages are not exposed and are thus not susceptible to tryptic cleavage.

Identification of the ATPase on the Basis of Differential Trypsin Sensitivity. If the above interpretation is correct, then it should be possible to use the phenomenon of differential trypsin sensitivity to identify the ATPase molecule in Na-DodSO$_4$–polyacrylamide gels. The experiment shown in Figure 1 demonstrates that this is the case. In this experiment, untreated membranes and membranes obtained in experiment 1 of Table I were subjected to NaDodSO$_4$–polyacrylamide gel electrophoresis and stained with Coomassie blue. Panel A shows the stained gel thus obtained and panel B shows densitometric scans comparing control and trypsin-treated membranes (wells 3 and 5), ATP-protected and trypsin-treated membranes (wells 4 and 5), and control and ATP-protected membranes (wells 3 and 4). Examination of these data reveals that only one protein, which runs as a broad band at M_r ~105 000 ± 5000, displays differential trypsin sensitivity. In the absence of MgATP, trypsin treatment results in the almost complete removal of this protein from the high molecular weight region of the gel, but in the presence of MgATP, only a small piece is removed, reducing the molecular weight of the protein to about 101 000. Taken together with the ATPase data in Table I, these results suggest that the 105 000-dalton protein is at least a subunit of the electrogenic ATPase and that removal of the 4000-dalton piece does not appreciably affect the ability of the enzyme to hydrolyze ATP. From these data alone, it cannot be concluded that the 105 000-dalton protein is the hydrolytic moiety of the ATPase because it could be a required subunit which does not contain the hydrolytic site. However, since it will be shown below that the 105 000-dalton protein is phosphorylated and dephosphorylated at a rate sufficient to account for all the ATP hydrolysis catalyzed by these membranes, we shall hereafter refer to this molecule as the ATPase.

Phosphorylation of the ATPase. Figure 2 demonstrates that the ATPase can be phosphorylated. Membranes obtained in experiment 2 of Table I were incubated with [γ-^{32}P]ATP, washed, disaggregated, and electrophoresed at pH 2.4 as described in the figure legend.[4] The resulting gel was then stained, destained, and subjected to autoradiography as described under Experimental Procedures. Panel A shows the stained gel and panel B shows the autoradiogram. Wells 1 represent control membranes, wells 2 represent membranes treated with trypsin in the presence of MgATP, and wells 3 represent membranes treated with trypsin in the absence of MgATP. Panel A shows that NaDodSO$_4$–polyacrylamide gel electrophoresis in the pH 2.4 system gives essentially the same results as the modified Laemmli system; only one protein (indicated by the arrow) displays differential trypsin sensitivity. In the absence of MgATP, trypsin treatment results in loss of this protein from the high molecular weight region of the gel, but in the presence of MgATP, only a small piece is removed, resulting in a slight shift downward. Molecular weight markers are not included for the pH 2.4 system because this system is not entirely reliable for the determination of the molecular weight of the ATPase. Nevertheless, the differential trypsin sensitivity of the protein indicated by the arrow and its relative abundance identify it as the 105 000-dalton protein and hence the ATPase. The autoradiogram in panel B shows

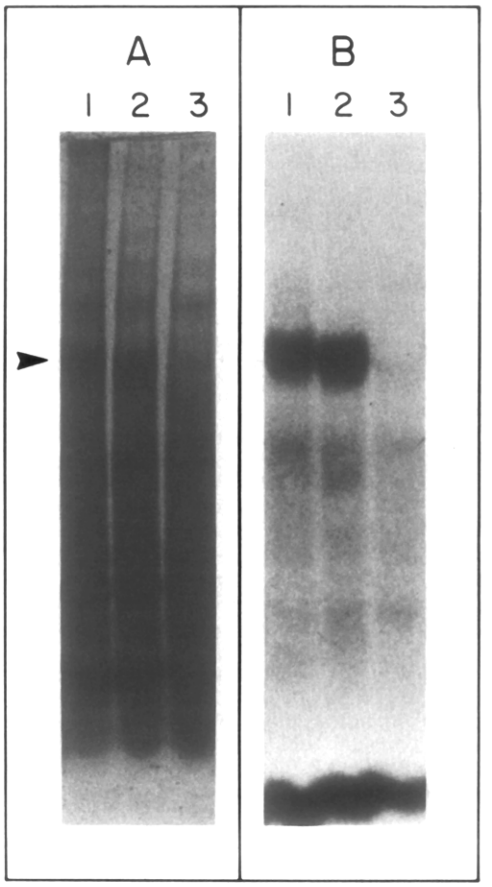

FIGURE 2: Phosphorylation of the ATPase with [γ-^{32}P]ATP. Plasma membrane vesicles from experiment II of Table I were labeled for 5 s at 30 °C with [γ-^{32}P]ATP in a reaction mixture containing 0.15 mL of vesicles [0.375 mg of protein in 0.01 M MES (pH 6.8 with Tris)] and 0.05 mL of 8 mM [γ-^{32}P]ATP–$MgSO_4$ (pH 6.8 with Tris; specific activity 150 Ci/mol). The reaction was stopped by the addition of 4 mL of ice-cold 0.3 M perchloric acid, and the membranes were pelleted by centrifugation at 1900*g* for 15 min. The membranes were washed twice by resuspension in 4 mL of 0.3 M perchloric acid containing 1 mM ATP–$MgSO_4$ and 5 mM NaH_2PO_4 and centrifuged at 1900*g* for 15 min. The resulting membrane pellet was disaggregated and subjected to electrophoresis (25 μg of protein/well) in the pH 2.4 NaDodSO$_4$–polyacrylamide gel electrophoresis system (5.6% resolving gel) as described under Experimental Procedures. The resulting gel was stained, destained, reswollen as described under Experimental Procedures, dried in a Bio-Rad gel dryer, and subjected to autoradiography for 213 h. Panel A shows the stained, dried gel, and panel B shows the autoradiogram. Well 1, control vesicles; well 2, vesicles treated with trypsin in the presence of MgATP; well 3, trypsin-treated vesicles. The arrow points to the ATPase.

that the ATPase is labeled by [γ-^{32}P]ATP. In membranes treated with trypsin in the absence of MgATP, the predominant ^{32}P-labeled protein is missing, and in membranes treated with trypsin in the presence of MgATP, the labeled protein shifts to a slightly lower molecular weight. Moreover, since the autoradiogram in panel B was obtained from the stained gel in panel A, the two can be overlayed and compared. When this was done (not shown), it could be seen that the predominant band in the autoradiogram corresponds exactly to the stained band indicated by the arrow and that both shift downward to an identical position in membranes treated with trypsin in the presence of MgATP. The band at the botton of the autoradiogram presumably represents lipid labeling since there is no corresponding Coomassie blue positive material in this region of the gel (panel A). Although this material may be of some future interest, it is disregarded in these studies because of its low turnover rate (see Figure 3). Finally, al-

[4] Because the phosphoryl-enzyme linkage is unstable under the conditions of the NaDodSO$_4$–polyacrylamide gel electrophoresis system described in Figure 1, the electrophoretic analysis was performed with the pH 2.4 NaDodSO$_4$–polyacrylamide gel electrophoresis system.

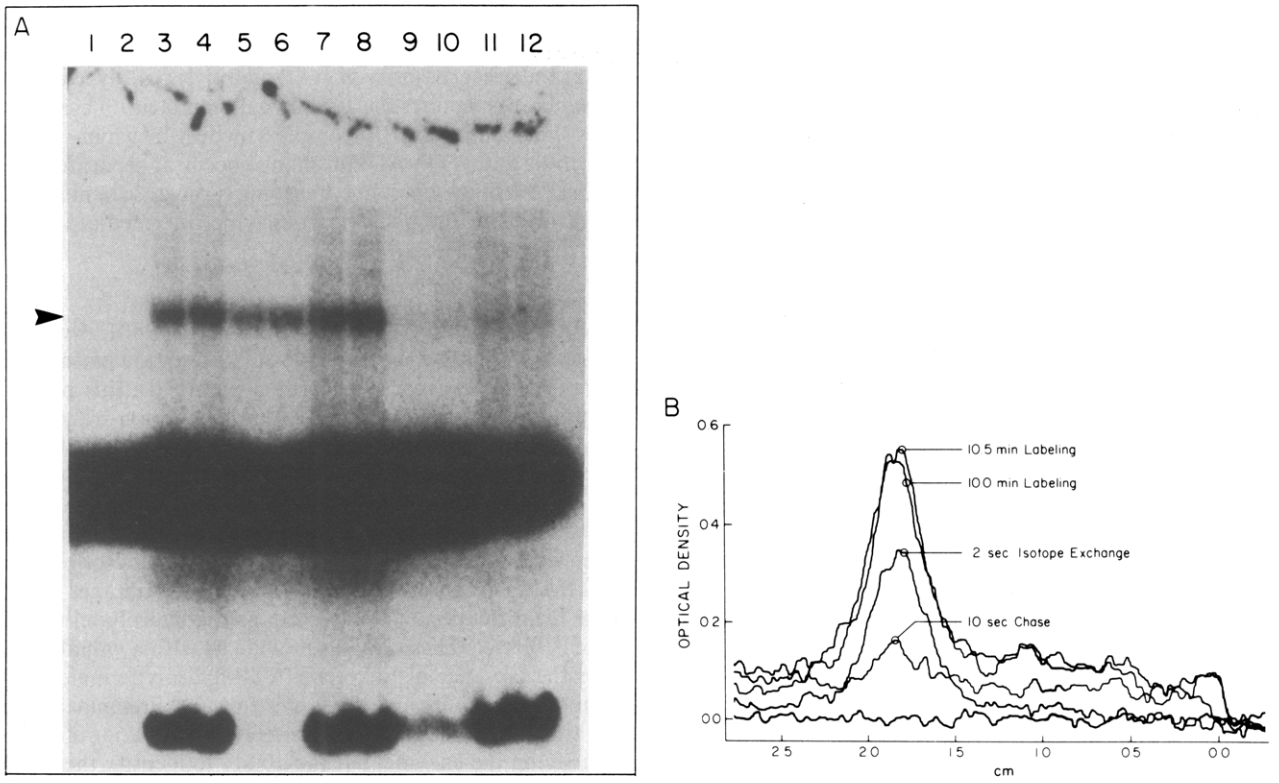

FIGURE 3: Rate of turnover of the phosphoryl-enzyme intermediate. Plasma membrane vesicles [125 μL of a suspension containing 0.46 mg of protein in 0.01 M MES (pH 6.8 with Tris)] were incubated at 0 °C[5] with [γ-^{32}P]ATP under the following reaction conditions. Wells 1 and 2, 5 mL of 0.3 M perchloric acid was added to the vesicle suspension followed by the addition of a reaction mixture containing 31.25 μL of 0.08 M [γ-^{32}P]ATP–$MgSO_4$ (pH 6.8 with Tris; specific activity 9.2 Ci/mol), 12.5 μL of 0.1 M NaN_3, 75 μL of 0.01 M MES (pH 6.8 with Tris), and 6.25 μL of H_2O; wells 3 and 4, a reaction mixture identical with that described for wells 1 and 2 was mixed with the vesicle suspension, and the reaction was allowed to proceed for 10 min with stirring, followed by the addition of 5 mL of 0.3 M perchloric acid; wells 5 and 6, a reaction mixture containing 11.25 μL of 0.2 M ATP–$MgSO_4$ (pH 6.8 with Tris), 11.25 μL of 0.1 M NaN_3, 55 μL of 0.01 M MES (pH 6.8 with Tris), and 22.5 μL of H_2O was added to the vesicles, and the reaction was allowed to proceed for 10 min. At that time, the following mixture was added with stirring: 19.7 μL of 0.01 M [γ-^{32}P]ATP–$MgSO_4$ (pH 6.8 with Tris; specific activity 114 Ci/mol), 1.25 μL of 0.1 M NaN_3, and 4.1 μL of 0.049 M MES (pH 6.8 with Tris). After 2 s, the reaction was terminated by the addition of 5 mL of 0.3 M perchloric acid; wells 7 and 8, vesicles were treated as in reactions 3 and 4 except that the reaction was allowed to proceed for 10.5 min before addition of the perchloric acid; wells 9 and 10, the following reaction mixture was mixed with the vesicle suspension and the reaction was allowed to proceed for 10 min before the addition of 5 mL of 0.3 M perchloric acid: 96.9 μL of 0.161 M [γ-^{32}P]ATP–$MgSO_4$ (pH 6.8 with Tris; specific activity 1.4 Ci/mol), 12.5 μL of 0.1 M NaN_3, and 15.6 μL of 0.048 M MES (pH 6.8 with Tris); wells 11 and 12, a reaction mixture identical with that described for wells 1 and 2 was mixed with the vesicle suspension, and the reaction was allowed to proceed for 10 min. At that time, a mixture of 2 mL of 0.01 M MES (pH 6.8 with Tris), 125 μL of 0.2 M ATP–$MgSO_4$ (pH 6.8 with Tris), 125 μL of 0.1 M NaN_3, and 250 μL of H_2O was added to the reaction vessel, followed 10 s later by 2.5 mL of 0.6 M perchloric acid. After the above labeling reactions had been carried out, the membranes were pelleted by centrifugation (1900g, 15 min), washed twice by alternate resuspension in 5 mL of 0.3 M perchloric acid containing 1 mM ATP–$MgSO_4$ and 5 mM NaH_2PO_4, centrifuged, and disaggregated for electrophoresis in the pH 2.4 $NaDodSO_4$–polyacrylamide gel electrophoresis system as described under Experimental Procedures. Disaggregated samples were subjected to electrophoresis (30 μg of protein per well, 7.5% acrylamide resolving gel), and the resulting gel was dried without staining and subjected to autoradiography for 87.5 h. The autoradiogram is shown in panel A and densitometer scans of wells 1, 3, 5, 7, and 11 of the autoradiogram in the region of the ATPase band are presented in panel B. The reference base line at an optical density equal to zero is the scan of well 1. Scans of wells 3, 7, 5, and 11 are labeled "10.0-min Labeling", "10.5-min Labeling", "2 sec Isotope Exchange", and "10 sec Chase", respectively. Aliquots of the same vesicle suspension used in this experiment were simultaneously used to measure the rate of vanadate-inhibitable ATP hydrolysis at 0 °C as described under Experimental Procedures. This activity was linear for at least 30 min and proceeded at a rate of 7.5 nmol mg^{-1} of protein min^{-1}.

though not shown here, when membranes are incubated with [α-^{32}P]ATP or [^{32}P]P_i and then treated as described in this experiment, no label is found in the area of the ATPase, which rules out binding (physiological or artifactual) as the source of the radioactivity in the band. The results of these experiments show that the ATPase is covalently labeled with ^{32}P from [γ-^{32}P]ATP. Because the ^{32}P associated with this molecule is relatively acid stable and base labile and is released by hydroxylamine (unpublished results), it is likely that the ^{32}P is present in an acyl phosphate linkage as is the case with certain other ATPases (Hokin et al., 1965; Bastide et al., 1973; Degani & Boyer, 1973; Nishigaki et al., 1974), but further experimentation is needed to identify the nature of the phosphoryl–enzyme bond.

Kinetics of Phosphorylation and Dephosphorylation of the ATPase. An indication of the rate of turnover of the ^{32}P label associated with the ATPase is shown in Figure 3. In this experiment, membranes were incubated with [γ-^{32}P]ATP under several different conditions, the reactions were stopped by the addition of perchloric acid, the membranes were washed, disaggregated, and electrophoresed in the pH 2.4 $NaDodSO_4$–polyacrylamide gel electrophoresis system, and the resulting gel was dried and subjected to autoradiography. Wells 1 and 2 contained zero time labeling controls quenched with perchloric acid prior to the addition of radioactive ATP. Labeling of the ATPase does not occur under these conditions, but a large spot due to radioactive ATP is apparent. This spot is always present in autoradiograms of gels dried prior to staining and destaining but is removed by the latter processes (see Figure 2). Wells 3 and 4 represent vesicles incubated with 10 mM [γ-^{32}P]ATP for 10 min whereas wells 7 and 8 are derived from similar 10.5-min incubations. The ATPase band

is indicated by the arrow. These wells show that enzyme labeling is at a steady-state level between 10 and 10.5 min. Wells 5 and 6 represent membranes that were incubated with nonradioactive ATP (10 mM) for 10 min and then pulsed with [γ-^{32}P]ATP for 2 s at the same final [γ-^{32}P]ATP concentration and specific activity as in the 10- and 10.5-min incubations. It can be seen that the ATPase labeling approaches the steady-state level during the 2-s pulse, indicating a rapid turnover of the phosphoryl-enzyme intermediate. Wells 9 and 10 represent membranes labeled as in wells 3 and 4 with the addition of a 52.5 mM excess of nonradioactive ATP, and wells 11 and 12 represent membranes labeled as in wells 3 and 4, followed by a 10-s chase with 10 reaction volumes of 10 mM nonradioactive ATP. The fact that ATPase labeling is markedly reduced in both cases verifies that the label is derived from [γ-^{32}P]ATP rather than any of the high specific activity contaminants known to be present in some preparations of ^{32}P-labeled nucleotides. Moreover, the chase data independently establish that the label in the ATPase band rapidly turns over. To quantify the rate of turnover of the phosphoryl-enzyme intermediate, three autoradiograms of increasing exposure time were prepared from the gel produced in the experiment of Figure 3 and the ATPase bands in wells 3–8 were analyzed by densitometry. Over the twofold exposure range examined (10.6–21.2% decay of the ^{32}P in the gel), the total number of isotopic decompositions occurring during autoradiography correlated in an approximately linear fashion with the integrated density of the ATPase band in the autoradiograms for all six wells, indicating that the band density is a reliable measure of the relative amounts of radioactivity associated with the ATPase in each well. By this method of measurement, enzyme labeling reaches 75% of the steady state in 2 s, which corresponds to a turnover time of 2.7 s at 0 °C.[5]

From the rate of turnover of the phosphoryl-enzyme intermediate, it is possible to calculate whether phosphorylation and dephosphorylation of the ATPase occurs at a rate sufficient to account for the overall ATPase activity of the membranes, if the number of participating ATPase molecules is known. Quantitative densitometry of Coomassie blue stained gels such as that shown in Figure 2A indicates that the ATPase molecules comprise 5–6% of the vesicle protein, which corresponds to about 0.5 nmol of the 105 000-dalton protein mg^{-1} of protein. With a phosphoryl-enzyme turnover time of 2.7 s, on the assumption that all the ATPase molecules are active, 0.5 nmol of ATPase can account for approximately 11 nmol of P_i released min^{-1}. The rate of orthovanadate-sensitive ATP hydrolysis, measured under the conditions of the labeling experiment described in Figure 3, was 7.5 nmol of P_i released mg^{-1} of protein min^{-1}, indicating that the turnover of the intermediate is more than sufficient to account for all the ATP hydrolysis catalyzed by the membranes. The apparent excess in the rate of phosphoryl-enzyme turnover could be misleading since some of the vesicles may be right side out (unpublished experiments). Vesicles of this orientation would contribute falsely to the densitometric estimate of active ATPase molecules but would not contribute to the measured rate of ATP hydrolysis. Alternatively, the excess turnover rate may reflect an ATP–ADP exchange reaction since the fate of the enzyme-bound phosphate (reaction with H_2O to form P_i or reaction with ADP to form ATP) could not be determined from these studies. It should be pointed out, however, that only the excess phosphoryl-enzyme turnover rate can be explained by such an exchange reaction because the differential trypsin sensitivity experiments of Table I and Figures 1 and 2 show that the great majority of ATP hydrolysis catalyzed by these membranes requires the 105 000-dalton protein. Thus, without invoking unnecessarily complex models in which ATP hydrolysis and ATP–ADP exchange occur at separate sites, at least 70% of the phosphoryl-enzyme turnover rate must reflect hydrolysis of the phosphoryl-enzyme intermediate with the formation of P_i.

[5] The experiment was carried out at ice-bath temperature in order to slow the reaction to a point where the turnover rate could be accurately measured.

Discussion

In the preceding paper (Scarborough, 1980), it was demonstrated that the electrogenic ATPase in the plasma membrane of *Neurospora* is a proton pump. In this paper, we identify the hydrolytic moiety of the proton-translocating ATPase as a 105 000-dalton protein in $NaDodSO_4$–polyacrylamide gels on the basis of its differential susceptibility to tryptic cleavage in the presence or absence of MgATP. The fact that the 105 000-dalton protein is phosphorylated and dephosphorylated at a rate sufficient to account for essentially all the ATP hydrolysis catalyzed by the membranes confirms the identification of this protein as the hydrolytic moiety of the ATPase. These studies provide us with a valuable assay for the presence of the intact ATP-hydrolytic moiety in attempts to solublize and purify this enzyme. Preliminary results in this regard indicate that proteolysis is a major obstacle to purifying the enzyme, but the ability to identify the ATPase in $NaDodSO_4$–polyacrylamide gels is facilitating the development of appropriate preventive measures. It should be mentioned that if the functional proton-translocating ATPase contains subunits other than the hydrolytic moiety, the participation of such subunits in the mechanism of proton translocation will be verified only upon the development of reconstitution methods.

Previous reports from this laboratory demonstrated the existence of four other acyl phosphate containing proteins in the *Neurospora* plasma membrane (Scarborough & Dame, 1978; Scarborough, 1978). It was suggested that one or more of these proteins may be related to the *Neurospora* plasma membrane ATPase, and they may well be, but it is clear from subsequent studies (unpublished experiments) and the experiments reported here that none of these acyl phosphate containing proteins can be identified as the ATPase. None of these proteins displays differential trypsin sensitivity, and none is present in sufficient quantity to account for even a small percentage of the ATP hydrolysis catalyzed by these membranes. The major difference between our previous studies and the experiments presented here is that in our earlier work the membranes were labeled in the presence of submicromolar concentrations of high specific activity [γ-^{32}P]ATP. At such low ATP concentrations, labeling of the ATPase is not detectable, which suggests that cooperative interactions between ATP and the enzyme may be necessary for labeling. In the present studies, the membranes were labeled with low specific activity [γ-^{32}P]ATP at concentrations in the millimolar range, near the K_m of the ATPase ($\sim$2.5 mM). Under these conditions, ATPase labeling predominates, and labeling of the minor acyl phosphate containing proteins is not detectable because of the low specific activity of the [γ-^{32}P]ATP and the relatively low amounts of these proteins. At intermediate ATP concentrations (30–100 μM), both the ATPase and the minor acyl phosphate containing proteins are detectable (unpublished experiments). The function of the minor acyl phosphate containing proteins remains the subject of speculation at present, but since they all have molecular weights higher than the ATPase ($\sim$110 000–200 000), they are probably not

proteolytic artifacts. Moreover, since the rate of phosphorylation and dephosphorylation of each of these proteins is different, they are probably not electrophoresis artifacts. It is conceivable that these proteins may be proenzyme forms of the ATPase, but more experimentation will be necessary to test this idea.

The existence of minor acyl phosphate bearing proteins in the plasma membrane of *Neurospora* and the fact that they are clearly not the principal ATPase molecule are pertinent to a recent report of the existence of several acyl phosphate containing proteins in a crude preparation of yeast membranes (Willsky, 1979). In that report, based primarily on the phosphate turnover time and the inhibition of labeling by orthovanadate, it was cautiously suggested that one or two of these proteins may represent the yeast plasma membrane ATPase. On the basis of our experience, the identity of these acyl phosphate containing proteins with the yeast plasma membrane ATPase is uncertain for at least two reasons. First, it is apparent from the calculations presented in this communication that the turnover time is essentially meaningless without knowledge of the number of enzyme molecules involved. For example, several of the minor acyl phosphate containing proteins in the *Neurospora* plasma membrane turn over at rapid rates, but as mentioned above, there are not enough of these enzyme molecules present to account for even a small percentage of the ATP hydrolysis catalyzed by these membranes. Second, orthovanadate inhibits labeling of several of the acyl phosphate containing proteins present in the *Neurospora* plasma membranes, but as shown here, most of the ATP hydrolysis is catalyzed by the 105 000-dalton protein, indicating that vanadate sensitivity is of questionable diagnostic utility in studies of this kind. Thus, in light of these considerations, it is not clear whether any of the acyl phosphate containing proteins detected in the yeast membranes represent the plasma membrane ATPase or whether they are all minor acyl phosphate containing proteins like those found in the *Neurospora* plasma membrane.

The existence of a phosphoryl-enzyme intermediate in the catalytic mechanism of a proton-translocating ATPase described in this paper and the preceding paper (Scarborough, 1980) presents us with an interesting mechanistic paradox. Prior to these studies, two major classes of ion-translocating ATPases have been investigated. The mitochondrial, bacterial, and chloroplast ATPases catalyze electrogenic proton translocation, and although hard sought, no phosphorylated intermediate has ever been detected (Kozlov & Skulachev, 1977). On the other hand, the animal cell transport ATPases catalyze Na^+/K^+, Ca^{2+}, or electroneutral K^+/H^+ translocation and have well-defined phosphorylated intermediates (Nishigaki et al., 1974; Degani & Boyer, 1973; Sachs, 1977). The formation of a phosphorylated intermediate in the catalytic cycles of the animal cell transport ATPases has been a central theme of models for the molecular mechanisms of these ion pumps (Cavieres, 1977; Hasselbach, 1978; Tada et al., 1978; Sachs, 1977) whereas the absence of a phosphorylated intermediate in the catalytic cycles of the mitochondrial, bacterial, and chloroplast ATPases has inspired alternative proposals for the mechanism of electrogenic proton pumps (Mitchell, 1974; Boyer, 1977; Kozlov & Skulachev, 1977). It has generally been assumed that these two classes of energy-transducing ATPases are fundamentally different and that electrogenic proton pumping involves a different mechanism than Na^+/K^+, Ca^{2+}, or H^+/K^+ pumping. However, our experiments with the *Neurospora* plasma membrane ATPase indicate that ATP-driven, electrogenic proton translocation can take place via a mechanism that involves a phosphoryl-enzyme intermediate. These considerations indicate that either two independent mechanisms exist for ATP-driven, electrogenic proton translocation or the mechanisms of all the ion-translocating ATPases are more similar than previously thought. With these possibilities in mind, comparative studies of the various classes of ion-translocating ATPases should eventually lead to a better understanding of ion pumps in general.

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