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Proton Translocation Catalyzed by the Electrogenic ATPase in the Plasma Membrane of *Neurospora*†

Gene A. Scarborough

ABSTRACT: ATP hydrolysis catalyzed by the plasma membrane ATPase (ATP phosphohydrolase, EC 3.6.1.3) located on the outer surface of functionally inverted plasma membrane vesicles isolated from the eukaryotic microorganism *Neurospora crassa* gives rise to the generation of an interior positive membrane potential ($\Delta\Psi$) [Scarborough, G. A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1485–1488]. The studies presented here demonstrate that the electrogenic ion in this process is H^+. In the presence of MgATP and the permeant anion SCN^-, isolated *Neurospora* plasma membrane vesicles catalyze the concentrative uptake of the ΔpH probe [^{14}C]imidazole, and this uptake is markedly inhibited by the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which demonstrates MgATP-dependent intravesicular acidification (ca. 2 pH units). MgATP-dependent [^{14}C]imidazole uptake (ΔpH generation) and MgATP-dependent [^{14}C]SCN^- uptake ($\Delta\Psi$ generation) exhibit identical saturation kinetics with respect to the concentration of MgATP and are inhibited in parallel by increasing concentrations of the electrogenic ATPase inhibitors orthovanadate and diethylstilbestrol, which indicates that $\Delta\Psi$ and ΔpH are generated by the same enzyme. The fluorescent pH indicator, fluorescein-labeled dextran, placed inside the vesicles during the isolation procedure, exhibits marked time-dependent fluorescence quenching upon the addition of MgATP and SCN^-, and the fluorescence response is reversed by orthovanadate, CCCP, and nigericin plus K^+, which independently demonstrates intravesicular acidification energized by the plasma membrane ATPase. The results of these experiments provide convincing evidence that the electrogenic ATPase in the plasma membrane of *Neurospora* is a proton pump.

It has been recognized for some time that the plasma membrane of the eukaryotic microorganism *Neurospora crassa* maintains a transmembrane electrical potential ($\Delta\Psi$) of approximately 200 mV (interior negative) (Slayman, 1965). On the basis of electrophysiological studies that correlated $\Delta\Psi$ with intracellular ATP levels, Slayman et al. (1970, 1973) proposed that $\Delta\Psi$ is generated by an electrogenic ATPase located in the plasma membrane. Upon the development of the concanavalin A method for isolating *Neurospora* plasma membranes (Scarborough, 1975), it became possible to demonstrate the existence of an ATPase in the *Neurospora* plasma membrane, and the biochemical properties of this enzyme were subsequently characterized (Scarborough, 1977; Bowman &

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Slayman, 1977). Concurrent studies with the isolated plasma membrane vesicles led to the direct demonstration that the ATPase is capable of generating a membrane potential (Scarborough, 1976). It was shown in these experiments that ATP hydrolysis, catalyzed by the ATPase located on the outer surface of functionally inverted plasma membrane vesicles, gives rise to the generation of an interior positive $\Delta\Psi$ that can be measured as MgATP-dependent [^{14}C]SCN^- uptake or MgATP-dependent 8-anilinonaphthalene-1-sulfonic acid (ANS)[1] fluorescence enhancement. The apparent K_m for MgATP with respect to ATP hydrolysis and [^{14}C]SCN^- uptake (ca. 2.5 mM) proved to be remarkably similar to that obtained for $\Delta\Psi$ generation in the electrophysiological studies (Slayman et al., 1973), and taken together, the experiments with intact cells and isolated plasma membrane vesicles clearly indicate that the *Neurospora* plasma membrane does contain an electrogenic ATPase.

Although it is clear that the *Neurospora* plasma membrane ATPase utilizes the free energy of ATP hydrolysis to generate $\Delta\Psi$ via charge translocation, little direct evidence is available as to the identity of the electrogenic ion. Slayman (1970) has shown that the time course of regeneration of $\Delta\Psi$ in postanoxic hyphae of *Neurospora* is similar to the time course of extracellular acidification in suspensions of postanoxic germinated conidia and on the basis of such observations has suggested that the *Neurospora* plasma membrane ATPase is a proton pump. Additional support for this notion comes from recent studies in this laboratory on calcium transport in the isolated *Neurospora* plasma membrane vesicles (Stroobant & Scarborough, 1979b). In these experiments, it was shown that the isolated plasma membrane vesicles catalyze MgATP-dependent Ca^{2+} uptake against a considerable concentration gradient. Comparisons of MgATP-driven $^{45}Ca^{2+}$ uptake and [^{14}C]SCN^- uptake with respect to the MgATP concentration dependence, the effects of inhibitors, and the nucleotide and divalent cation specificities indicated that the energy for Ca^{2+} accumulation is derived from ATP hydrolysis catalyzed by the electrogenic plasma membrane ATPase. It was also shown that energized Ca^{2+} uptake is markedly inhibited by the proton conductor CCCP and by nigericin in the presence of K^+, which led to the conclusion that the immediate driving force for Ca^{2+} accumulation is a transmembrane pH gradient. The most reasonable interpretation of these findings is that the electrogenic ATPase generates a pH gradient that energizes a Ca^{2+}/H^+ antiporter (Mitchell, 1973).

While these experiments strongly suggest that the electrogenic ATPase translocates protons, ATP-driven proton translocation has not yet been demonstrated. The studies reported in this communication directly demonstrate ATP-dependent transmembrane proton movements in isolated *Neurospora* plasma membrane vesicles by two independent methods and essentially rule out the participation of other ions in the generation of $\Delta\Psi$. The results of these experiments leave little doubt that the electrogenic ATPase in the plasma membrane of *Neurospora* is a proton pump.

Experimental Procedures

Isolation of Neurospora Plasma Membrane Vesicles. Plasma membrane vesicles were isolated essentially as described by Stroobant & Scarborough (1979a) except that the methyl α-mannoside was recrystallized from hot (85 °C) water before use, and the "resuspension buffer" was 0.01 M MES adjusted to pH 6.8 with Tris. FD (5 mg/mL) was included during the methyl α-mannoside step to obtain vesicles containing FD. The FD–methyl α-mannoside solution was centrifuged (12000*g*, 30 min) and filtered (0.8 μm, Millipore) prior to use.

Standard Uptake Assay. [^{14}C]SCN^- (60 Ci/mol) and [^{14}C]imidazole (3.5 Ci/mol) uptakes were measured essentially as described by Scarborough (1976) except that the wash solution was 10 mM MES adjusted to pH 6.8 with Tris. Incubations were started by the addition of membranes.

Fluorescence Measurements. FD fluorescence was measured at 90° in a Perkin-Elmer Hitachi MPF-2A recording spectrophotofluorometer (direct mode) with a Farrand interference filter (peak wavelength, 515 nm; half-bandwidth, 14 nm; 40% transmission) in the path of the emission beam. The samples were stirred magnetically. The excitation wavelength was 475 nm, and light emission was monitored at 519 nm. A fluorescence of 100% was arbitrarily set at the top of the chart by varying the emission slit width, and 0% fluorescence was defined as the light emission that was not quenched at pH 4.8.

Determination of Intravesicular Water Content. A portion (3 mL) of a plasma membrane vesicle suspension (approximately 1 mg of protein/mL in 10 mM MES adjusted to pH 6.8 with Tris) was mixed with 80 μL of [^{3}H]H_2O (1 mCi/mL) and 60 μL of inulin[^{14}C]carboxylic acid solution (66 mg/mL, 1.7 mCi/g) and allowed to stand on ice for 15 min. Aliquots of this mixture (0.3 mL in duplicate) were then brought to 24 °C for 1 min, mixed with 48 μL of H_2O and 12 μL of 0.3 M $MgSO_4$–ATP (pH 6.8 with Tris), incubated for 1 min, and centrifuged for 3 min at top speed in a Beckman microfuge (ca. 9400*g*). The resulting membrane pellets were then suspended in H_2O and assayed for protein, and the ^{3}H and ^{14}C contents were determined by liquid scintillation counting in the cocktail of Patterson & Greene (1965). Intravesicular water content was calculated by subtracting the extravesicular water space (inulin[^{14}C]carboxylic acid space) from the total water space ([^{3}H]H_2O space). The value obtained from 24 different determinations was 3.05 ± 0.42 μL/mg of protein (mean ± SD). Determinations in the presence of 50 μM sodium orthovanadate or 40 μM KSCN did not differ significantly from the above value.

Other. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. ATPase activity was measured essentially as described by Scarborough (1977).

Materials. MES, Tris, bovine serum albumin, ATP (Tris salt, low in vanadate), ADP (Tris salt), EGTA, methyl α-mannoside, and diethylstilbestrol were purchased from Sigma. [^{14}C]KSCN, [^{3}H]H_2O, and inulin[^{14}C]carboxylic acid were from New England Nuclear, and [^{14}C]imidazole was from California Bionuclear Corp. CCCP was from Calbiochem, and FD (M_r 60 000–90 000) was from Polysciences. Sodium orthovanadate, analyzed as described by Stroobant & Scarborough (1979b), was from Fisher. All other chemicals were of reagent grade or of the highest purity obtainable from commercial sources.

Results

MgATP-Dependent Imidazole Uptake. Proton translocation catalyzed by the plasma membrane ATPase located on the outer surface of functionally inverted membrane vesicles should result in acidification of the intravesicular space. An interior acid pH gradient (ΔpH) in closed membrane systems can be

[1] Abbreviations used: ANS, 8-anilinonaphthalene-1-sulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FD, fluorescein-labeled dextran.

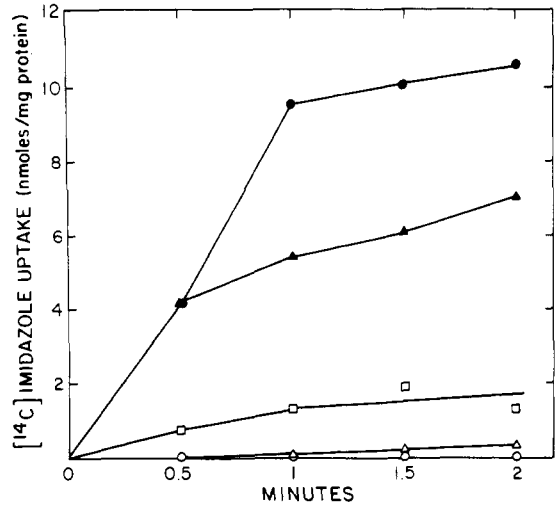

FIGURE 1: MgATP-dependent [^{14}C]imidazole uptake by *Neurospora* plasma membrane vesicles. Standard uptake assay. Incubations contained 25 μL of plasma membrane vesicle suspension (1.04 mg of protein/mL in 10 mM MES, pH 6.8 with Tris), 5 μL of 0.5 mM [^{14}C]imidazole, and the indicated additions made up to a total volume of 50 μL with water. Additions were adjusted to pH 6.8 with Tris and then made to give the following final concentrations: (○) ADP and $MgSO_4$ at 10 mM each, or ADP, $MgSO_4$, and NaSCN at 10 mM each, or ADP, $MgSO_4$, and NaSCN at 10 mM each and EGTA at 0.5 mM, or ATP and $MgSO_4$ at 10 mM each; (△) ATP and $MgSO_4$ at 10 mM each and EGTA at 0.5 mM; (▲) ATP, $MgSO_4$, and NaSCN at 10 mM each; (●) ATP, $MgSO_4$, and NaSCN at 10 mM each and EGTA at 0.5 mM; (□) ATP, $MgSO_4$, and NaSCN at 10 mM each, EGTA at 0.5 mM, ethanol at 0.5% (v/v), and CCCP at 50 μM. Ethanol at this concentration had no effect.

monitored by measuring the distribution of weak bases such as methylamine (Rottenberg et al., 1972), fluorescent amines (Schuldiner et al., 1972), ammonia (Rottenberg & Grunwald, 1972), and imidazole (Sachs, 1977). If the unprotonated form of any weak base is permeant and the protonated form is much less permeant, the molecule will accumulate on the acid side of the membrane (Rottenberg et al., 1972). Methylamine (and presumably ammonia) is not accumulated by the *Neurospora* plasma membrane vesicles under any conditions yet tried, probably because the protonated form is too permeant and results with the fluorescent amines have been equivocal. However, energized imidazole uptake can readily be demonstrated. Figure 1 shows the uptake of [^{14}C]imidazole by the *Neurospora* plasma membrane vesicles under a variety of conditions. There is no detectable uptake in the presence of MgADP (open circles) or MgATP (also open circles). However, in the presence of MgATP and the permeant anion SCN^- (Schuldiner et al., 1974), imidazole uptake is markedly stimulated (closed triangles). The effect is seen in the presence of KSCN or NaSCN but not K_2SO_4 or Na_2SO_4, which indicates that the stimulation is due to SCN^- (data not shown). Because SCN^- has no effect on uptake in the presence of MgADP (open circles), its effect is clearly on energized imidazole uptake. Figure 1 also shows the effect of the Ca^{2+} chelator EGTA on imidazole uptake. The addition of EGTA to assays containing MgATP elicits a slight stimulation (open triangles), and in assays containing MgATP plus SCN^-, EGTA elicits a considerable enhancement of imidazole uptake (closed circles). EGTA has no effect on uptake in the presence of MgADP plus SCN^- (open circles), which indicates that its effect is likewise on energized imidazole accumulation. The probable bases for the stimulatory effects of SCN^- and EGTA will be discussed below. Figure 1 also shows the effect of the protonophore CCCP (Harold, 1972) on energized imidazole uptake. If imidazole uptake is a measure of ΔpH generation,

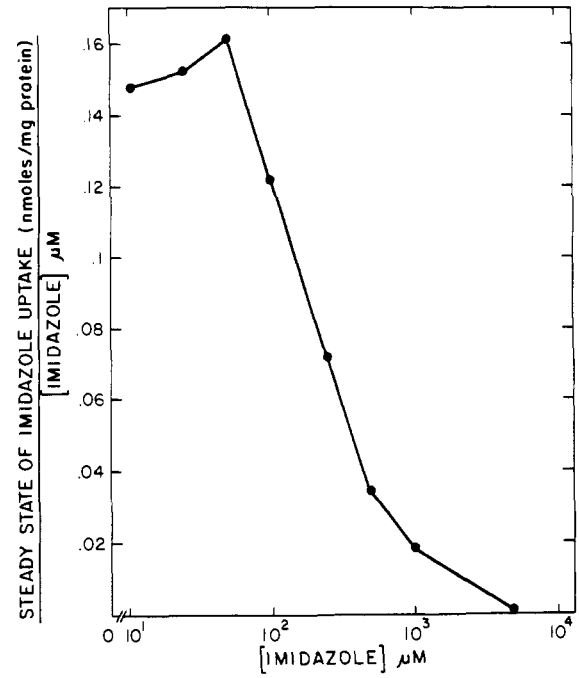

FIGURE 2: Imidazole accumulation ratio as a function of the imidazole concentration. Standard uptake assay. Incubations contained 25 μL of plasma membrane vesicle suspension (0.96 mg of protein/mL in 10 mM MES, pH 6.8 with Tris), 5 μL of 0.1 M $MgSO_4$–ATP (pH 6.8 with Tris), 5 μL of 0.1 M NaSCN, 5 μL of 5 mM EGTA (pH 6.8 with Tris), 5 μL of H_2O, and 5 μL of the various [^{14}C]imidazole solutions at concentrations between 0.1 and 50 mM. Assays were performed at 0, 30, 60, 90, and 120 s, and the steady state of imidazole uptake was determined from the lines of best fit plotted from the uptake data.

the process should be inhibited by CCCP. It can be seen that MgATP-dependent imidazole uptake in the presence of SCN^- and EGTA is inhibited greater than 80% by CCCP (open squares). These results thus demonstrate intravesicular acidification dependent upon MgATP, which indicates the existence of a proton-translocating ATPase in the *Neurospora* plasma membrane. Taking a value of 3 μL of intravesicular space per mg of protein (see Experimental Procedures), MgATP-dependent imidazole uptake in the presence of SCN^- and EGTA represents a ΔpH of about 2 units, assuming that the pK_a of imidazole is 6.92. This represents a minimum value because some of the vesicles may be right side out.

Weak bases used to monitor ΔpH will report falsely low values of ΔpH when used in excess (Rottenberg et al., 1972). Therefore, the imidazole accumulation ratio was measured as a function of the imidazole concentration to maximize the usefulness of imidazole as a probe of ΔpH in these membranes. Figure 2 describes the results of this experiment. It can be seen that the imidazole accumulation ratio is essentially constant in the concentration range between 10 and 50 μM and then decreases at higher imidazole concentrations. This experiment establishes the optimum practical concentration of imidazole to be used (50 μM) and also indicates that imidazole at this concentration does not affect the ΔpH it is being used to monitor.

The results described thus far provide support for the existence of a proton-translocating ATPase in the *Neurospora* plasma membrane, and because proton translocation catalyzed by this enzyme is markedly stimulated by the permeant anion SCN^-, it was considered likely that the proton-translocating ATPase is the enzyme that generates $\Delta\Psi$. To gain additional support for this contention MgATP-dependent $\Delta\Psi$ generation ([^{14}C]SCN^- uptake) and MgATP-dependent ΔpH generation

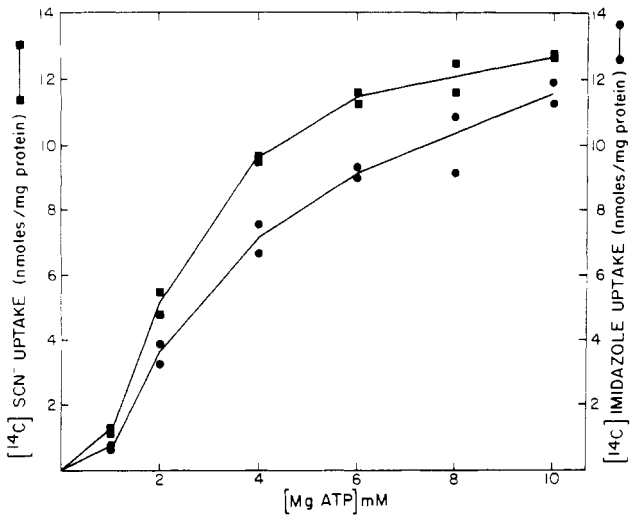

FIGURE 3: MgATP-dependent [^{14}C]SCN^- and [^{14}C]imidazole uptakes as a function of the MgATP concentration. Standard uptake assay, 2-min incubations. (■) [^{14}C]SCN^- uptake: the assay mixtures contained 25 μL of plasma membrane vesicle suspension (1.26 mg of protein/mL in 10 mM MES, pH 6.8 with Tris), 5 μL of 0.4 mM [^{14}C]KSCN, 5 μL of 5 mM EGTA (pH 6.8 with Tris), 10 μL of H_2O, and 5 μL of the various MgATP solutions (ATP and $MgSO_4$ at equimolar concentrations, adjusted to pH 6.8 with Tris) at concentrations between 10 and 100 mM. (●) [^{14}C]Imidazole uptake: the assay mixtures contained 25 μL of plasma membrane vesicle suspension (1.26 mg of protein/mL in 10 mM MES, pH 6.8 with Tris), 5 μL of 0.5 mM [^{14}C]imidazole, 5 μL of 0.1 M NaSCN, 5 μL of 5 mM EGTA (pH 6.8 with Tris), 5 μL of H_2O, and 5 μL of the various MgATP solutions at concentrations between 10 and 100 mM.

([^{14}C]imidazole uptake) were compared with respect to their dependence upon the concentration of MgATP and the effect of inhibitors. Figure 3 shows MgATP-dependent [^{14}C]SCN^- uptake and MgATP-dependent [^{14}C]imidazole uptake measured as a function of the concentration of MgATP. It can be seen that the two processes depend upon MgATP in an identical fashion, which indicates that $\Delta\Psi$ and ΔpH are generated by the same enzyme. Orthovanadate and diethylstilbestrol have previously been shown to be potent inhibitors of ATP hydrolysis (Bowman et al., 1978) and MgATP-dependent $\Delta\Psi$ generation (Stroobant & Scarborough, 1979b) in the isolated *Neurospora* plasma membranes. Figure 4 compares the effects of increasing concentrations of orthovanadate and diethylstilbestrol on MgATP-dependent [^{14}C]-SCN^- uptake and MgATP-dependent [^{14}C]imidazole uptake. It can be seen that both processes are affected by the two inhibitors in an extremely similar fashion. These results, together with the results described in Figure 3, leave little doubt that $\Delta\Psi$ and ΔpH are generated by the same ATPase. This, of couse, means that the electrogenic ATPase is a proton pump.

Fluorescence Measurements with FD-Containing Vesicles. Intravesicular pH changes in the *Neurospora* plasma membrane vesicles can also be monitored with another pH probe, fluorescein-labeled dextran (FD). FD is an indicator of pH in the pH range between 5 and 8. The fluorescence intensity of this molecule is maximal around pH 8 and almost totally quenched at pH 5. This probe has been used to monitor intralysosomal pH changes (Ohkuma & Poole, 1978) and is a useful pH indicator in biological systems provided that it can be incorporated into the compartment of interest. With the *Neurospora* plasma membrane vesicles, this is straightforward because the membranes are isolated as topologically open sheets and are subsequently converted to closed vesicles. Inclusion of FD during the ghost-to-vesicle transition results

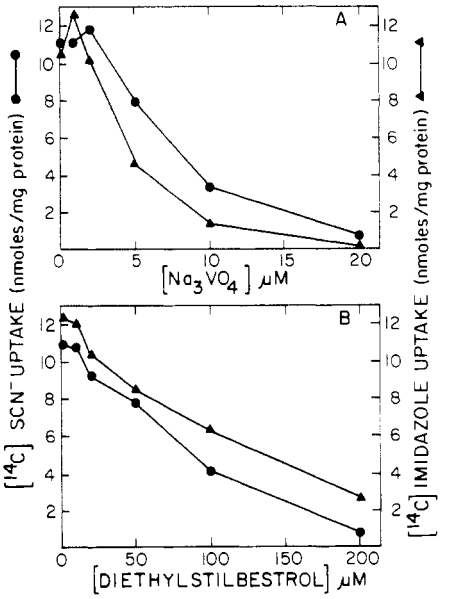

FIGURE 4: The effects of orthovanadate and diethylstilbestrol on MgATP-dependent [^{14}C]SCN^- and [^{14}C]imidazole uptakes. Standard uptake assay, 2-min incubations, points indicate the average of duplicate determinations. (●) [^{14}C]SCN^- uptake: the assay mixtures contained 25 μL of plasma membrane vesicle suspension (0.96 mg of protein/mL in 10 mM MES, pH 6.8 with Tris), 5 μL of 0.4 mM [^{14}C]KSCN, 5 μL of 5 mM EGTA (pH 6.8 with Tris), 5 μL of 0.1 M $MgSO_4$–ATP (pH 6.8 with Tris), 5 μL of H_2O, and for the vanadate curve, an additional 5 μL of H_2O or 5 μL of the various Na_3VO_4 solutions at concentrations between 10 and 200 μM. For the diethylstilbestrol curve, the assays contained membranes, [^{14}C]KSCN, EGTA, and MgATP as above and, in addition, 10 μL of H_2O and 0.25 μL of ethanol or 0.25 μL of the various diethylstilbestrol solutions in ethanol at concentrations between 2 and 40 mM. (▲) [^{14}C]Imidazole uptake: the assay mixtures contained 25 μL of plasma membrane vesicle suspension (0.96 mg of protein/mL in 10 mM MES, pH 6.8 with Tris), 5 μL of 0.5 mM [^{14}C]imidazole, 5 μL of 0.1 M NaSCN, 5 μL of 5 mM EGTA (pH 6.8 with Tris), 5 μL of 0.1 M $MgSO_4$–ATP (pH 6.8 with Tris), and the inhibitors as described above for [^{14}C]SCN^- uptake.

in the trapping of this probe in the intravesicular space. Figure 5 shows the fluorescence responses obtained with FD-containing vesicles under a variety of conditions. In trace A, it can be seen that the addition of ATP to the vesicles (with Mg^{2+} present) results in a small, immediate decrease in fluorescence intensity followed by a time-dependent fluorescence quenching which levels off in about 1 min. Subsequent addition of the permeant anion SCN^- results in an additional, rapid quenching of fluorescence. The addition of orthovanadate, which inhibits the ATPase, returns the fluorescence response to near the original level, indicating that the majority of the fluorescence quenching is reversible and dependent upon ATP hydrolysis catalyzed by the plasma membrane ATPase. In trace B, the steps of trace A were repeated, but the protonophore, CCCP, was added in place of vanadate. Vanadate was then added after the fluorescence response had stabilized. The time-dependent fluorescence quenching is inhibited 50–60% by CCCP, and the response remaining is abolished by vanadate. The reversal of the fluorescence response induced by CCCP indicates that much of the time-dependent fluorescence quenching is due to intravesicular acidification. The inability of CCCP to completely reverse the fluorescence quenching is discussed below. If the time-dependent fluorescence quenching is indeed due to MgATP-dependent intravesicular acidification, the process should also be reversed by nigericin, which catalyzes an electroneutral exchange of H^+ for K^+ (Pressman, 1976), in the presence of K^+. The experiment shown in trace C demonstrates that this is the case. In this experiment, the

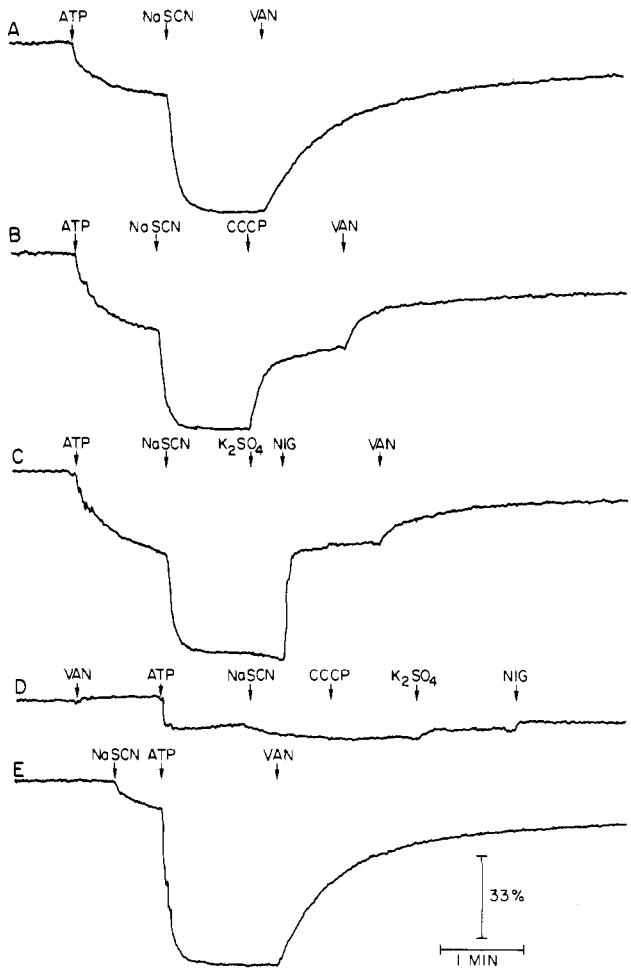

FIGURE 5: MgATP-dependent FD fluorescence quenching. FD-containing plasma membrane vesicles (0.94, 0.64, 0.46, 0.51, and 0.69 mg of protein for traces A–E, respectively) were resuspended in 1.71 mL of 10 mM MES containing 0.53 mM EGTA and 10.53 mM $MgSO_4$ (adjusted to pH 6.8 with Tris), brought to 24 °C, and transferred to a cuvette in the spectrophotofluorometer, and the light emission was adjusted to 100% by varying the emission slit width. Where indicated, the following additions were made: ATP, 90 μL of 0.2 M ATP adjusted to pH 7.5 with Tris; NaSCN, 4 μL of 4.5 M NaSCN; VAN, 20 μL of 4.5 mM Na_3VO_4; CCCP, 4 μL of 22.5 mM CCCP in ethanol; K_2SO_4, 25 μL of 0.72 M K_2SO_4; NIG, 2 μL of 22.5 mM nigericin in dimethyl sulfoxide. Equivalent additions of ethanol or dimethyl sulfoxide had no effect on the fluorescence quenching. ATPase activity (see Discussion) was determined in a similar incubation containing the membrane suspension (0.53 mg of protein), ATP, and NaSCN as above and 5 mM NaN_3 to inhibit traces of mitochondrial ATPase activity (Bowman et al., 1978).

order of addition was ATP, NaSCN, then K_2SO_4 followed quickly by nigericin, and then vanadate. It can be seen that nigericin in the presence of K^+ abolishes most of the time-dependent fluorescence quenching, and the response remaining is eliminated by vanadate. Although not shown, in a similar experiment, the addition of nigericin alone was about one-third as effective in reversing the fluorescence response as was nigericin plus K^+. The observation that nigericin without K^+ has some effect is not surprising because nigericin has a reasonable affinity for Na^+ (Pressman, 1968), and this ion is added with the SCN^- in these experiments. The effect of nigericin plus K^+ corroborates the results obtained with CCCP, further indicating that the MgATP-dependent fluorescence quenching is the result of intravesicular acidification. Trace D shows a control experiment. In this experiment, vanadate was added first, followed by ATP, NaSCN, CCCP, K_2SO_4, and nigericin. The results indicate that the immediate, small decrease in fluorescence intensity observed upon the addition of ATP is probably not dependent upon ATP hydrolysis and that all of the other additions do not nonspecifically perturb the FD fluorescence. Finally, in trace E, NaSCN was added first, followed by ATP and then vanadate. This was done so as to obtain an estimation of the maximum rate of the full fluorescence response. The implications of this experiment will be discussed below.

Is Another Ion Translocated before H^+? Although the experiments described above clearly demonstrate intravesicular acidification dependent upon ATP hydrolysis catalyzed by the electrogenic plasma membrane ATPase, it is necessary to consider the possibility that some other ion is translocated first, followed by proton antiport or symport into the vesicles. For example, X^+ translocation into the vesicles followed by X^+/H^+ antiport could result in the generation of an interior positive $\Delta\Psi$ and intravesicular acidification. Likewise, X^- translocation out of the vesicles followed by X^-/H^+ symport would have the same result. A consideration of the components of the electrogenic pump assay medium ($[^{14}C]SCN^-$ uptake) indicates that the most likely candidates for such mechanisms are Mg^{2+} (added with the ATP), K^+ (added with the $[^{14}C]SCN^-$), traces of Cl^- (present during the ghost-to-vesicle conversion), and possible trace contaminants such as Ca^{2+} (present in many commercial ATP preparations) and Na^+. The possibility that any of these ions is the true electrogenic species is highly unlikely for the following reasons. Co^{2+} can replace Mg^{2+} as the divalent cation in the $[^{14}C]SCN^-$ uptake assay, but no $^{57}Co^{2+}$ uptake is detectable, which essentially rules out Mg^{2+}. The assay is unaffected by the Ca^{2+} chelator EGTA, which rules out Ca^{2+}. The assay works well in vesicles prepared in the absence of Cl^-, which eliminates this ion, and under the conditions of the $[^{14}C]SCN^-$ uptake assay, there is no detectable uptake of $^{86}Rb^+$ (a K^+ analogue) or $^{22}Na^+$, which essentially rules out these ions as the electrogenic species. Therefore, although an unknown trace contaminant ion is not totally eliminated as the electrogenic species, by far the most reasonable interpretation of all the experimental results presented here is that MgATP-dependent generation of $\Delta\Psi$ and ΔpH occurs as a result of proton translocation catalyzed by the electrogenic plasma membrane ATPase.

Discussion

The results presented in this paper demonstrate by two independent methods that the electrogenic ATPase in the plasma membrane of *Neurospora* is a proton pump. In the presence of MgATP and the permeant anion SCN^-, isolated *Neurospora* plasma membrane vesicles catalyze concentrative uptake of the ΔpH probe [^{14}C]imidazole. MgATP-dependent imidazole uptake and MgATP-dependent $\Delta\Psi$ generation display identical saturation kinetics with respect to the concentration of MgATP and are inhibited in parallel by increasing concentrations of the electrogenic ATPase inhibitors orthovanadate and diethylstilbestrol, indicating that concentrative imidazole uptake is energized by the electrogenic ATPase. These results, together with the fact that MgATP-dependent imidazole uptake is markedly inhibited by the proton conductor CCCP, indicate that energized imidazole uptake is a reflection of proton translocation catalyzed by the electrogenic plasma membrane ATPase.

MgATP-dependent imidazole uptake is markedly stimulated by the permeant anion SCN^-. This effect can be explained if the extent of proton translocation catalyzed by the ATPase is limited by $\Delta\Psi$. SCN^- at the concentration used in these experiments (10 mM) collapses $\Delta\Psi$ (Stroobant & Scarborough, 1979b), which should allow additional proton translo-

cation leading to an increase in ΔpH and a stimulation of imidazole uptake. Such postulated proton movements are actually demonstrated in the FD fluorescence experiments of Figure 5. In the absence of SCN^-, there is some ΔpH generation, and the addition of SCN^- increases the magnitude of ΔpH approximately threefold. The inability to detect significant amounts of energized imidazole uptake in the absence of SCN^-, even though some ΔpH is generated under these conditions, is probably due to expulsion of the imidazole cation by $\Delta\Psi$.

Energized imidazole uptake is also stimulated by EGTA. The stimulatory effect of EGTA is reversed by Ca^{2+} in 100 μM excess (data not shown), and since EGTA does not enhance the rate of ATP hydrolysis or electrogenic pumping (as measured by MgATP-dependent [^{14}C]SCN^- uptake) (data not shown), its effect most likely is to prevent dissipation of ΔpH by Ca^{2+} which is present as a contaminant in the assays. Partial dissipation of ΔpH by Ca^{2+} is not surprising since Ca^{2+} is accumulated by the vesicles and the immediate driving force for Ca^{2+} accumulation is ΔpH (see above and Stroobant & Scarborough, 1979b).

The FD fluorescence experiments independently demonstrate proton translocation catalyzed by the electrogenic ATPase. The addition of ATP (in the presence of Mg^{2+}) to vesicles containing the pH indicator FD gives rise to time-dependent fluorescence quenching that is markedly stimulated by the addition of SCN^-. The MgATP-dependent fluorescence response is abolished by orthovanadate, which indicates that the fluorescence quenching is a manifestation of ATP hydrolysis catalyzed by the electrogenic plasma membrane ATPase. The reversal of MgATP-dependent fluorescence quenching by the proton conductors CCCP and nigericin plus K^+ indicates that the fluorescence quenching is due to intravesicular acidification and, hence, proton translocation catalyzed by the electrogenic ATPase. The apparent discrepancy between the effects of CCCP on FD fluorescence quenching (ca. 50–60% reversal) and imidazole uptake (>80% inhibition) is misleading. It can be calculated from the amount of imidazole uptake remaining in the presence of CCCP (Figure 1) that a ΔpH of approximately 1 unit still exists under these conditions. With this in mind, since the full fluorescence quenching response presumably represents a ΔpH of approximately two units, CCCP should reverse only half of the fluorescence response, which is the observed effect. The reason for the apparent discrepancy is that imidazole accumulation is a logarithmic function of ΔpH whereas FD fluorescence is roughly proportional to intravesicular pH in the pH range of these experiments (Ohkuma & Poole, 1978). These results indicate that CCCP is simply unable to fully dissipate the ΔpH generated by the vesicles under these experimental conditions. More importantly, the consistency in the effects of CCCP on imidazole uptake and FD fluorescence provides evidence that both methods are reliable indicators of transmembrane proton movements.

The extent of [^{14}C]imidazole uptake at the steady state provides a reasonably accurate estimate of the magnitude of the ΔpH that the proton-translocating ATPase is capable of generating in these membranes. However, because the rate of imidazole uptake may well depend on the rate of penetration of the uncharged imidazole molecule, imidazole uptake is not reliable as a means of measuring the rate of proton translocation catalyzed by the ATPase. However, the rate of FD fluorescence quenching does not depend upon the rate of permeation of any species other than H^+, with the possible exception of SCN^-. Thus, even if the rate-limiting step in MgATP-dependent FD fluorescence quenching is the rate of SCN^- permeation, the initial rate of the fluorescence response seen upon the addition of ATP in the presence of Mg^{2+} and SCN^- (Figure 5E) provides a minimum estimate of the rate of proton translocation catalyzed by the ATPase. Titration of the vesicles[2] indicates that the total buffering capacity of the vesicles is approximately 300 nmol of H^+ mg^{-1} of protein per 2 pH units in the pH range of 6.8–4.8. Assuming that one-half of this buffering capacity is due to the inside surface of the vesicles and their contents, the initial rate of fluorescence quenching (ca. 6 s/2 pH units) corresponds to a proton-translocation rate of 1500 nmol of H^+ mg^{-1} of protein min^{-1}. The ATP hydrolysis rate under the conditions of the FD experiment was determined to be 673 nmol of P_i liberated mg^{-1} of protein min^{-1}, which suggests an approximate stoichiometry (H^+/ATP) of 2. While this approach provides an experimental framework for the future determination of an accurate stoichiometry, these results are only approximate and are presented here to make a different point. Although ATP hydrolysis, $\Delta\Psi$ generation, and ΔpH generation all exhibit identical saturation kinetics with respect to the concentration of MgATP (Figure 3 and Scarborough, 1976), it was conceivable that electrogenic proton translocation and overall ATP hydrolysis could be catalyzed by different proteins with identical saturation kinetics. Because the isolated *Neurospora* plasma membranes appear to have associated proteolytic activity (unpublished observations), it was considered possible that the enzyme that catalyzes the majority of ATP hydrolysis could be a nonpumping proteolytic fragment of the intact pump. The fact that the rates of proton translocation and overall ATP hydrolysis are similar precludes this possibility and indicates that bulk ATP hydrolysis and electrogenic proton translocation are catalyzed by the same molecule.

With the physiological role of the *Neurospora* plasma membrane ATPase now firmly established, we have turned our attention to an investigation of the molecular mechanism of proton translocation catalyzed by this enzyme. Progress in this regard is described in the following paper (Dame & Scarborough, 1980) wherein we identify the hydrolytic moiety of the ATPase in sodium dodecyl sulfate–polyacrylamide gels and demonstrate that the enzyme undergoes phosphorylation and dephosphorylation during its catalytic cycle.

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[2] In this experiment, FD-containing vesicles were prepared as described under Experimental Procedures except that 6.7 mM Na_2SO_4 was used as the "resuspension buffer". The vesicle suspensions (0.32 and 0.42 mg of protein, respectively, in 1.71 mL of 6.7 mM Na_2SO_4) were adjusted to pH 6.8 with 20 mM NaOH and then titrated to pH 4.8 with 0.005 N H_2SO_4. Similar titrations were also carried out with 1.71 mL of 6.7 mM Na_2SO_4, and the total buffering capacity of the vesicles was calculated from the difference.

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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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