Electrogenic Proton Translocation Coupled to ATP Hydrolysis by the Plasma Membrane Mg^{2+}-dependent ATPase of Yeast in Reconstituted Proteoliposomes*

The purified plasma membrane Mg^{2+}-dependent ATPase of the yeast Schizosaccharomyces pombe was incorporated in liposomes using a cholate-dialysis method. The ATPase activity of the incorporated enzyme was stimulated by the H^{-}-conducting agent carbonyl cyanide m-chlorophenylhydrazone and to a much lower extent by the K^{+}/H^{+} ionophore valinomycin in the presence of potassium. The K^{+}/H^{+} exchanger nigericin (plus K^{+}) did not stimulate ATPase activity, whereas the combined addition of both nigericin plus valinomycin (plus valinomycin). Moreover, during the steady state ATP hydrolysis, a H^{+} entry was again observed when the membrane potential was collapsed upon addition of valinomycin in the presence of K^{+}.

These data demonstrate that the plasma membrane ATPase of yeast cells is involved in electrogenic H^{+} translocation coupled to ATP hydrolysis since the purified enzyme incorporated in the liposomes is virtually free of mitochondrial F,F_{0}-ATPase contaminant.

In the yeast Schizosaccharomyces pombe a plasma membrane Mg^{2+}-dependent ATPase has been well characterized in its membrane-bound form (1-3) as well as after solubilization with L-α-lysophosphatidylcholine (2, 3-7). Similar ATPases have been isolated from other fungi and yeast (8, 9). In several respects, these enzymes resemble the plasma membrane Mg^{2+}-dependent (Na^{+}, K^{+})-ATPase of animal cells. However, the fungi/yeast ATPase has been proposed to work as an electrogenic H^{+} pump (10-13) which generates an electrochemical proton gradient across the plasma membrane in contrast with the well characterized (Na^{+}, K^{+})-ATPase which generates electrochemical Na^{+} plus K^{+} gradients (14).

The early suggestions that the fungi/yeast plasma mem-

brane ATPase was involved in proton translocation were based on observations obtained in whole cells (10-13). In such complex systems, unequivocal interpretation of the experimental data was not possible.

However, a more direct approach was provided by Scarp observed (15, 16) using inside-out vesicular preparations of the plasma membrane from Neurospora crassa where the generation of a membrane potential and of a pH gradient coupled to ATP hydrolysis was observed. Nevertheless, the fungi plasma membrane has a variety of carriers which might be responsible for proton translocation (i.e. cation/H^{+} antiporter(s)). Therefore, it was difficult to exclude the possibility that the observed pH gradient in the inside-out vesicles resulted from the coupled functions of a cation(s)-pumping ATPase and some cation/H^{+} antiporter(s) working in series via (a) common intermediate cation(s). In such systems, a net movement of the intermediate cation(s) would not be detected.

In this report, we describe the electrogenic proton translocation carried out by a fairly pure plasma membrane ATPase (free of mitochondrial F,F_{0}-ATPase contaminant) of the yeast S. pombe in reconstituted proteoliposomes. The ATPase activity of the reconstituted enzyme is strongly stimulated by the H^{+}-conducting agent CCCP or by an association of the K^{+}/H^{+} carrier nigericin plus the K^{+}-ionophore valinomycin in the presence of potassium.

Proton movement in the external medium was directly monitored with a pH-electrode. Upon addition of MgATP, a fast proton uptake took place when the charge-compensating cation potassium (+ valinomycin) was present. Moreover, during steady state ATP hydrolysis, a fast and transient proton uptake was also observed when the generated membrane potential was collapsed upon addition of valinomycin in the presence of potassium.

These results definitively demonstrate that the plasma membrane Mg^{2+}-dependent ATPase of yeast cells carries out electrogenic proton translocation.

**MATERIALS AND METHODS**

**Chemicals**—ATP disodium salt (grade II) or magnesium salt, egg L-α-lysophosphatidylcholine, soybean L-α-phosphatidylcholine (II-S or IV-S types), cholic acid (sodium salt), bovine serum albumin (fraction V), and CCCP were purchased from Sigma Chemical Co. Valinomycin was obtained from Calbiochem, and nigericin (batch 477-19B) was a kind gift of Dr. J. W. Westley, The Roche Institute, Nutley, NJ.

**Culture Condition**—S. pombe 972h was grown at 30°C in aerobic

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1 The abbreviations used are: CCCP, carbonyl cyanide m-chlorophenylhydrazone; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid.

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conditions in 5.8% (w/v) glucose, 2% (w/v) yeast extract at pH 4.5. The cells were harvested in exponential phase of growth.

Purification of Plasma Membrane—The washed cells were homogenized with glass beads in a MSK Braun homogenizer and the plasma membrane was purified by a selective acid precipitation step as previously described (4) except that the acid precipitation was carried out at pH 4.8 and the subsequent sucrose gradient was omitted.

Solubilization and Purification of the ATPase—The typical procedure derived from that previously reported (4) was as follows. Sixty mg of purified plasma membrane were suspended in 240 ml of 10 mM Tris/acetate, 1 mM ATP, and 1 mM EDTA at pH 7.5. 480 ml of L-lysophosphatidylcholine were added and incubated for 10 min at 15 °C under strong magnetic stirring. The mixture was centrifuged at 160,000 × g for 45 min. The supernatant was concentrated to a final volume of 12 ml either with Amicon CF50A cones using a Sorvall SW 27 rotor at 4 °C, each tube was fractionated into 32 portions of 1.2 ml. The three fractions of highest ATPase activity were pooled and concentrated to 2 to 3 ml with the Amicon CF50A cones. The final suspension which contained an average of 1.4 ± 0.5 mg of protein (11 preparations) was stored at -70 °C until use. After thawing, the average specific ATPase activity of the 11 preparations was 12 ± 5 μmol of ATP hydrolyzed/min/mg of protein.

Analytical Procedure—Protein concentration was determined by the method of Lowry et al. (17) using bovine serum albumin as a standard. Inorganic phosphate was determined as described by Pullman and Penefsky (18). Proton movements were measured with a highly sensitive small diameter combination pH glass electrode (A. H. Thomas Co., model 4094-L25) in a 3 ml chamber thermostated at 25 °C. The electrode outputs were amplified through a Beckman Expandomatic SS-2 pH-meter and fed into a dual channel Tekman Electronics Ltd. recorder adjusted to adequate chart speed. Known amounts of standard solutions of HCl and KOH were added to calibrate the pH-electrode response in all the experiments.

Incorporation of Purified Plasma Membrane ATPase in Liposomes—The cholate-dialysis method of Kagawa and Racker (19) was used. A typical procedure was as follows: to 0.6 ml of the purified plasma membrane ATPase preparation containing 0.3 to 0.8 mg of protein were added 3 or 4 ml of 50 mM KCl, 50 mM NaCl, 10 mM MgCl₂, 50 mM K-HEPES at pH 7.0. In the experiments where H⁺ movements were measured, only 3 mM HEPES was used at pH 7.0 or pH 6.2. The phospholipid was added at a final concentration of 1.5 to 2% (w/v) and the cholate at a final concentration of 1.0 to 1.3% (w/v).

The mixture maintained at 0 °C was sonicated with a micro tip for about 10 min with frequent on/off periods until total clarification was obtained. When necessary, the pH was readjusted after sonication. The sonicated mixture was dialyzed against 500 ml of the same medium for 26 h at 4 °C with three buffer changes. In all the experiments, the external medium had the same ionic composition that the medium used for the preparation of the proteoliposomes.

Electron Microscopy—The proteoliposomes were fixed with 1% (w/v) osmium tetroxide and negatively stained with 2% (w/v) uranyl acetate. The samples were dried on a Formvar carbon-coated grid and were observed in a Philips 301 electron microscope at 60 kV. The average diameter of the liposome was 400 Å and no multilayer structures were observed.

Mitochondrial F₁ ATPase Preparation—The mitochondrial F₁ ATPase was solubilized by chloroform treatment of submitochondrial particles of S. pombe and purified as previously described (20).

Anti-F₁ Serum Preparation and Immunoprecipitation Assay—Anti-F₁ serum was obtained after repeated injection of rabbits with the purified yeast mitochondrial F₁-ATPase. The immunoprecipitation assay was carried out in a 2% (w/v) agarose gel as previously described (21).

Electrophoresis—Polyacrylamide gel (11.5% (w/v)) electrophoresis was performed in the presence of 0.1% (w/v) sodium dodecyl sulfate using a slab gel as described by Laemmli (22).

RESULTS

Purity of the Plasma Membrane ATPase Preparations—The purified plasma membrane ATPase used for reconstituted activity was obtained by a scaled up procedure derived from that previously described (4). Polyacrylamide gel electrophoresis of these preparations in the presence of sodium dodecyl sulfate also shows a single major band of an apparent molecular weight of 100,000 (Fig. 1A, run 3) as previously described (4, 20). None of the mitochondrial F₁-ATPase components (Fig. 1A, run 1) were detected in the run of the purified plasma membrane ATPase (Fig. 1A, run 3). However, the apparently homogenous major band of 100,000 daltons observed in polyacrylamide electrophoresis could be separated in at least two components by isoelectric focusing (Fig. 1B). Unexpectedly, the ATPase activity of this purified preparation was partly sensitive to oligomycin (Table 1). Average inhibitions of 22 and 38% were observed at pH 6.0 and 7.0, respectively. Similar
Effect of oligomycin on different forms of the plasma membrane ATPase

For all the experiments except the ones with incorporated enzyme, the ATPase activity was assayed for 8 min at 30 °C in 1 ml of 25 mM K-MES or K-HEPES, 12 mM MgCl₂, 6 mM ATP (sodium salt) at the indicated pH. The experiments with incorporated enzyme were carried out in 50 mM NaCl, 50 mM KCl, 50 mM Na-HEPES, 10 mM MgCl₂, 10 mM ATP (sodium salt). Methanol 0.5% (v/v) was present in all the assays. When indicated, 100 μg of L-α-lysophosphatidylcholine was added to the assay mixture. Twenty-five μg of oligomycin was used in all the experiments except in the first ones with the membrane-bound enzyme where 40 μg was used. The reaction was started by addition of 5 to 20 μg of protein or 4.5 mg of phospholipid liposomes with 25 μg of incorporated enzyme. The values given are the mean ± S.D. The number of experiments done are indicated in parentheses.

| Form of enzyme       | pH of assay | Inhibition by oligomycin % |
|----------------------|-------------|----------------------------|
| Membrane-bound       | 6.0         | 5 ± 5 (13)                 |
| Solubilized + lysolcehlin | 6.0        | 22 ± 8 (10)               |
| Solubilized + lysolcehlin | 7.0        | 38 ± 19 (8)               |
| Solubilized, no lysolcehlin | 7.0       | 28 (1)                    |
| Incorporated         | 7.0         | 4 ± 1 (2)                  |

oligomycin sensitivity was observed when the ATPase activity was measured in the absence of added L-α-lysophosphatidylcholine. In contrast to the purified enzyme, the sensitivity of the membrane-bound ATPase to oligomycin was very low (see Table I). When the purified enzyme was incorporated in liposomes, negligible sensitivity to oligomycin was detected. The inhibition by oligomycin was observed only at a high concentration of the inhibitor (25 μg of oligomycin·ml⁻¹). At lower concentration a clear stimulation of about 20% of the ATPase activity was observed (results not shown).

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These results prompted a further search for possible mitochondrial F₁,F₀-ATPase contaminants in our preparations. When the ATPase was assayed at pH 9.0 (optimum for the mitochondrial ATPase) negligible activity was found. Moreover, the ATPase activity was totally insensitive to 2.5 mM sodium azide, an inhibitor for the mitochondrial enzyme (23). However it was up to 95% inhibited by 100 μg vanadate, an inhibitor for the plasma membrane enzyme (23). Furthermore, the purified plasma membrane ATPase did not precipitate in an immunodiffusion assay in the presence of a rabbit antiserum prepared against the S. pombe mitochondrial F₁-ATPase (results not shown). Fig. 2 shows that the plasma membrane ATPase activity was insensitive to the mitochondrial F₁-antiserum. In contrast, the mitochondrial enzyme was fully inhibited by the antiserum. Thus, the preparations of purified plasma membrane ATPase used in this study were virtually free of mitochondrial F₁,F₀-ATPase contaminant.

Recovery of the ATPase Activity after Incorporation into Liposome Membrane—The solubilized plasma membrane ATPase was incorporated into the liposomes using the cholate-dialysis method (19). Table I shows that after incorporation 31% of the total ATPase activity was present in the proteoliposomes. This recovery went up to 49% after uncoupling of the ATPase activity by the addition of CCCP.

Assuming random incorporation of all the ATPase molecules into the liposome membrane, it was expected that only 50% of the ATPase molecules would expose their catalytic site to the external medium and be able to carry out hydrolysis of the externally added substrate MgATP. If this was the case, virtually no inactivation of the ATPase activity would have taken place during the reconstitution procedure.

ATP Hydrolysis Control by the Electrochemical Ion(s) Gradient(s)—If the incorporated ATPase carries out energy-dependent ion(s) translocation across the proteoliposomes membrane, an increase of the ATPase activity is expected to occur when the chemical ion(s) gradient(s) and/or the electrical membrane potential are collapsed. Such stimulation is shown in Fig. 3, which presents the time course of ATP hydrolysis by the incorporated plasma membrane ATPase in liposomes in the presence and absence of the H⁺-conducting agent, CCCP. Valinomycin and nigericin (in the presence of K⁺) were also tested for their capacity to stimulate the rate of ATP hydrolysis in the incorporated enzyme. As shown in Table III, no significant effects by CCCP, valinomycin, or nigericin were observed in the control experiments carried out with the nonincorporated enzyme assayed in the presence of L-α-lysophosphatidylcholine. However, in the incorporated enzyme, a strong stimulation was given by CCCP (61%) and by valinomycin plus nigericin in the presence of potassium (42%). In both cases, the proteoliposome membranes are freely permeable to protons. On the other hand, the H⁺/K⁺ electro-neutral exchanger nigericin alone does not produce any significant effect (11%). Valinomycin alone which makes the proteoliposome membrane freely permeable to K⁺ stimulates by only 19%. The stimulation by CCCP was also observed in the presence of valinomycin or nigericin (44 and 56%, respectively). When the incorporation and ATPase assay were car-
Hydrolysis in the presence of uncoupler

The ATP hydrolysis control ratio induced by CCCP was not measured of proton permeability of the membrane by imposing direct measurement of proton permeability of the membrane by imposing an artificial ΔpH in the presence of the charge-compensating cation potassium (+ valinomycin) confirmed the low H⁺ conductance of the membranes. As expected, CCCP increased proton conductance by at least 3 orders of magnitude (results not shown).

Effect of Temperature on the Degree of Coupling—Change of fluidity of the lipid bilayer could in principle modify the permeability of the liposome membrane to proton and/or to other ions. Such change of permeability would influence the degree of stimulation of the incorporated ATPase activity by protonophores. Fig. 4A shows that the ATPase activity progressively decreased when the assay temperature was lowered from 30–10 °C in the presence as well as in the absence of CCCP. However, the per cent of stimulation induced by the uncoupler on the rate of ATP hydrolysis increased dramatically at lower temperature. This indicates that at low temperature the conductance of the membrane to protons was decreased. The effect of CCCP on the interaction of the ATPase molecule with the phospholipid bilayer was also studied. The Arrhenius plots of the ATPase activity in the presence and in the absence of CCCP gave parallel lines indicating that the activation energy is not modified by the uncoupler. Both plots exhibited an identical breaking point at 19 °C (Fig. 4B). The ATP hydrolysis and the ion(s) translocation functions are thus similarly affected by the fluidity of the lipid bilayer suggesting further that these two processes are tightly coupled.

**Table III**

| Experiment | Form of enzyme | pH | Addition | ATPase activity | Relative activity |
|------------|----------------|----|----------|-----------------|-----------------|
| A          | Solubilized    | 7.0| None     | 15.8% CCCP      | 6%              |
|            |                |    | CCCP     | 16.8            | 6               |
|            |                |    | Valinomycin | 15.6            | 14              |
|            |                |    | Nigericin | 16.7            | 5               |
| B          | Incorporated   | 7.0| None     | 3.6% CCCP       | 6               |
|            |                |    | CCCP     | 6.1             | 12              |
|            |                |    | Valinomycin + nigericin | 4.3 | 19 |
|            |                |    | Nigericin | 4.0             | 11              |
|            |                |    | CCCP + valinomycin | 5.2 | 44 |
|            |                |    | CCCP + nigericin | 5.4             | 50              |
| C          | Incorporated   | 6.2| None     | 2.6% CCCP       | 6               |
|            |                |    | CCCP     | 5.5             | 111             |
|            |                |    | Valinomycin + nigericin | 4.7 | 81 |

**Fig. 3.** Time course of the hydrolysis of ATP by the reconstituted ATPase in the presence and in the absence of CCCP. The ATPase assay was carried out at 30 °C during the indicated periods of time in a final volume of 1 ml in 50 mM KCl, 50 mM NaCl, 10 mM MgCl₂, 10 mM ATP (sodium salt), 50 mM K-HEPES, pH 7.0, 0.2% (v/v) methanol. Where indicated, 4 µM CCCP was added. The reaction was started upon addition of proteoliposomes (2.72 mg of phospholipids + 17.1 µg of incorporated protein).

**Fig. 4.** Effect of temperature on the rate of ATP hydrolysis by the reconstituted ATPase in the presence and in the absence of uncoupler. A, the ATPase activity was assayed at the indicated temperature for 10 min in a final volume of 1 ml in 50 mM KCl, 50 mM NaCl, 10 mM MgCl₂, 10 mM ATP (sodium salt), 50 mM K-HEPES, pH 7.0, 0.2% (v/v) methanol in the presence (●—●) and in the absence (○—○) of 4 µM CCCP. The reaction was started upon addition of proteoliposomes (2.72 mg of phospholipid + 17.1 µg of incorporated protein). The same plot also indicates the per cent of stimulation by CCCP on the rate of ATP hydrolysis (■—■). B, Arrhenius plot of the data presented in part A.
Electrogenic proton translocation by the incorporated plasma membrane ATPase induced upon addition of ATP. In A, proteoliposomes (29.1 mg of phospholipid + 150 µg of incorporated protein) in a total volume of 2 ml were incubated at 25 °C for about 30 s in the presence of 1 mM valinomycin in 50 mM KCl, 50 mM NaCl, 10 mM MgCl₂, 3 mM K-HEPES, pH 6.2. A pulse of 1 mM MgATP was added and proton movement recorded. Control addition of potassium as charge-compensating cation. This electrogenic proton movement was not seen when valinomycin was omitted. The dotted line in the same figure indicates the absence of proton movement when MgATP was added to the medium in the absence of liposomes. However, experiments carried out with liposomes without incorporated ATPase showed same artifactual alkalization not related to ATPase activity upon addition of MgATP. This proton’s disappearance is probably due to a physicochemical interaction between MgATP and the phospholipid membrane. Fig. 5B shows other series of control experiments. As expected, the presence of CCCP avoided almost completely the electrogenic H⁺ influx induced by MgATP in the presence of K⁺ (+ valinomycin). When vanadate (a specific inhibitor for the plasma membrane ATPase) was present, only the artifactual alkalization previously mentioned was observed upon addition of MgATP. As in the first series of traces, the dotted line in Fig. 5B indicated the absence of proton movement upon addition of the substrate to the medium without liposomes. The above experiments demonstrated that electrogenic protons were pumped by the plasma membrane ATPase. This conclusion was further supported by another experimental approach. Fig. 6 shows that during steady state ATP hydrolysis, the addition of valinomycin in the presence of K⁺ as charge-compensating cation, induced a fast and transient proton entry. In the presence of the H⁺/K⁺ exchanger nigericin, addition of valinomycin (in the presence of K⁺) does not induce a net proton influx because H⁺ and K⁺ were recycling under these conditions. Moreover, in the absence of ATP, no proton movement was observed upon addition of valinomycin. This control experiment also shows that the continuous acidification observed in previous traces (before the addition of valinomycin) during steady state ATP hydrolysis was due to a scalar proton release from the phosphate liberated in the external medium. Electrogenic proton translocation was also observed not only when the experiments were carried out at pH 6.2 (as in Fig. 6) but also at pH 7.0 (results not shown).

In the experiments of Figs. 5 and 6 where ATP hydrolysis took place in the presence of valinomycin (+ K⁺), it was expected that subsequent addition of CCCP would discharge the generated pH gradient. However, under such conditions, a net proton influx (rather than efflux) was observed (result not shown). The explanation for this proton influx was provided by the experiments shown in Fig. 7. When valinomycin alone was added to proteoliposomes or to simple liposomes (in the presence of K⁺), a very slow H⁺ influx was observed (Fig. 7A). Subsequent addition of CCCP induced an extremely fast H⁺ entry. When the order of addition was inverted, the proton entry took place only when both reagents were present. Moreover, nigericin alone induced similar proton influx. These experiments point out that the interior of the liposomes was more alkaline than the external medium. Similar results were obtained when the proteoliposomes were prepared at pH 6.2. This pH gradient, generated during the formation of the liposomes, was probably due to the protonation of the inner face of the lipid bilayer, which is favored by the small intraliposome volume and/or by the low buffer capacity. Smaller pH gradients were found when the liposomes were prepared in higher buffer capacity medium (result not shown).

Fig. 7B presents a plot of the extent of H⁺ movement induced by nigericin at different external pH values. Proton exit was observed at pHₘₑₐₙ above 7.75 and proton entry was observed below external pH 7.75. The experiments clearly shows that when pHₘₑₐₙ = pHₘₑₓₑ, no net proton movements took
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**Fig. 7** Determination of the intraliposomal pH. A, 7.5 mg of phospholipid liposomes were incubated at 25 °C in 2 ml of 50 mM KCl, 50 mM NaCl, 10 mM MgCl\(_2\), 3 mM K-HEPES, pH 7.0. Where indicated, 1 µg·ml\(^{-1}\) valinomycin, 2 µM CCCP, or 1 µg·ml\(^{-1}\) nigericin was added and proton influx was recorded. B, plot of the total extent of protein movement upon addition of nigericin to the liposome in the same conditions as in A but at different external pH. The point of intersection of the sigmoidal curve with the abscissa determines the intraliposomal pH.

place upon addition of nigericin and that the pH\(_{\text{in}}\) (intersection with abscissa) was higher than the pH used for the preparation of the liposomes (pH = 7.0). This proton gradient (alkaline inside) was also observed when the liposomes were prepared at pH 6.2. This proton gradient should favor the electrogenic proton influx coupled to ATP hydrolysis. The fact that after ATP hydrolysis in the presence of potassium (+ valinomycin) the total intraliposomal space was still more alkaline that the external medium could indicate that a large proportion of the liposomes are free of an active incorporated ATPase molecule.

**DISCUSSION**

The involvement of plasma membrane-bound ATPases in the generation of electrical potential and the asymmetric distribution of different ions across the cell membrane became evident after the chemiosmotic principles were formulated in the early sixties (24). In animal cells, the (Na\(^+\), K\(^+\))·ATPase is the major electrogenic pump and is responsible for the asymmetric distribution of these cations across the plasma membrane (14). However, in plant cells as well as in yeast/fungi cells, the plasma membrane ATPase has been proposed to be involved in proton translocation (see reviews 25, 26). The most direct evidence for such model came from recent work of Scarborough demonstrating the generation of a membrane potential (positive inside) as well as a proton gradient (acidic inside) in inside-out plasma membranes vesicles from *N. crassa*, during ATP hydrolysis (15, 16). However, a definitive proof of proton translocation by the plasma membrane ATPase can only come from studies in a reconstituted system with a purified enzyme where interfering reactions with other membrane-bound proton carriers are avoided. This has been achieved in the present work with the purified plasma membrane ATPase of *S. pombe*. Although the oligomycin sensitivity of the purified plasma membrane ATPase activity was in principle disturbing, our results clearly exclude any contamination by the mitochondrial F\(_0\)·F\(_1\)-ATPase. Moreover, the (Na\(^+\), K\(^+\))·ATPase of animal cells also has been shown to have some sensitivity to oligomycin (27). In addition, the plasma membrane ATPase of plants appears to be also sensitive to this inhibitor (28).

The purified plasma membrane ATPase of *S. pombe* was incorporated into liposomes which were of an average diameter of 400 Å and free of multilayer structures. In this system, ATP is hydrolyzed at the external side of the membrane and a proton influx is expected to generate a membrane potential positive inside during ATP hydrolysis. This electrogenic proton movement was monitored using a combination pH-glass electrode in medium of low buffer capacity. At the present time, this technique is the most reliable one for measuring proton fluxes in membrane vesicles.

The electrogenic nature of the H\(^+\) pumping by the ATPase was demonstrated because the observed H\(^+\) translocation only took place in the presence of the charge-compensating cation K\(^+\) (+ valinomycin). The present study does not rule out that in addition to H\(^+\), other cations could also be pumped in the opposite direction by the same ATPase. If this was the case, the stoichiometric ratio H\(^+\)/cation should be higher than 1 to account for the net electrogenerosity of the reconstituted pump and the membrane potential of living cells. Potassium appears to be a good candidate for such function because cells are able to build up huge concentration gradients of this cation across the cell membrane. Further work is obviously needed to check this possibility. Determination of the H\(^+\)/ATP ratio is also required before a definitive model for the plasma membrane ATPase function can be provided. The isoelectrofocusing of the purified plasma membrane ATPase separates at least two different components from the single 100,000 molecular weight band obtained in polyacrylamide gel electrophoresis. The question whether or not these two components are both incorporated into the liposomes and are both required for the ATP hydrolysis and/or the coupled ion(s) translocation(s) is presently under study.

Another interesting observation made in this paper is the control of the rate of ATP hydrolysis by the electrochemical H\(^+\) gradient generated across the proteoliposome membrane. The rate of ATP hydrolysis was stimulated by more than 100% upon addition of uncouplers which collapse the electrochemical proton gradient. This corresponds to an ATP hydrolysis control ratio of more than 2. In *vivo*, the formation of an electrochemical proton gradient is expected to slow down the rate of ATP hydrolysis in order to avoid over-consumption of ATP by the plasma membrane ATPase which may be one of the major ATP-consuming pathways of living cells.

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