

Reconstitution of the Proton-translocating Adenosine Triphosphatase of Yeast Plasma Membranes*

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The plasma membrane ATPases of eukaryotic cells of the vegetable type (fungi, plants, and algae) have been postulated to operate as proton pumps which generate membrane potentials and drive the uptake of nutrients by proton co-transport (Poole, R. J. (1978) *Annu. Rev. Plant Physiol.* 29, 437-460). In order to verify this important physiological role, a purified preparation of the yeast plasma membrane ATPase has been reconstituted with soybean phospholipids by a freeze-thaw-sonication procedure. The reconstituted proteoliposomes catalyzed a $^{32}\text{P}_i$ -ATP exchange partially sensitive to proton ionophores (uncouplers) and to the proton-potassium exchange carrier nigericin. The reaction was completely inhibited by the nonspecific ionophore gramicidin and by the combination of uncouplers with the potassium ionophore valinomycin. These results are interpreted as evidence for two types of proton transport catalyzed by the enzyme preparation: electrogenic proton transport and electroneutral proton-potassium exchange.

The existence of electrogenic proton pumps in the plasma membranes of fungi, plants, and algae has been postulated on the basis of two lines of evidence. Firstly, membrane potentials measured with microelectrodes cannot be explained in terms of passive ion diffusion potentials and the effect of metabolic inhibitors on the potentials and on ion fluxes suggest the existence of an electrogenic proton efflux powered by ATP (1). Secondly, the active transport of nutrients in fungi (2, 3), plants, and algae (1) occurs by co-transport with protons, suggesting a chemiosmotic mechanism where the electrochemical proton gradient generated by the pump is the driving force for nutrient uptake (4). This hypothetical proton pump would be the physiological equivalent to the (Na, K)-ATPase¹ of animal plasma membranes, an enzyme which generates Na^+ and K^+ gradients and electrical potentials in animal cells and drives the co-transport of nutrients with Na^+ (5). There-

fore, the nature of this proton pump has become one of the most crucial issues in the bioenergetics of the vegetable type of eukaryotic cells.

Plasma membrane ATPases with similar kinetic properties have been identified in fungi (6-9) and plants (10) and the enzymes from *Schizosaccharomyces pombe* (11) and *Saccharomyces cerevisiae* (12) have been purified. Although there is circumstantial evidence about the identification of this ATPase with the proton pump (13-17), this important point should be established by demonstrating ATP-dependent proton transport in proteoliposomes reconstituted with the purified enzyme (18). One manifestation of the ion-pumping activity of membrane ATPases is the catalysis of a $^{32}\text{P}_i$ -ATP exchange sensitive to ionophores (19, 20). In this process, the enzyme can be considered to operate alternatively in both directions of the reaction: firstly, ATP hydrolysis generates an ion gradient and then this gradient drives ATP synthesis. In the present work, we communicate the reconstitution of the purified yeast plasma membrane ATPase into liposomes catalyzing a $^{32}\text{P}_i$ -ATP exchange dependent on the proton gradient.

MATERIALS AND METHODS

Enzyme Purification—Yeast plasma membrane ATPase was purified from commercial baker's yeast by a modification² of our previously described procedure (12). Briefly, plasma membranes were isolated from the homogenate by sucrose gradient centrifugation and washed with 0.5 M guanidine. The residue was solubilized with the detergent Zwittergent TM 314 and the ATPase was purified by glycerol gradient centrifugation and a final guanidine treatment. The preparation had a specific activity of 17-22 $\mu\text{mol}/\text{min} \times \text{mg}$ (30 °C and pH 5.5) and, as previously described (12), contained a major subunit of 100,000 daltons.

Reconstitution of Proteoliposomes—The purified ATPase was diluted to 0.4 mg/ml in a medium containing 10 mM Tricine, 1 mM EDTA, 2.8 mM 2-mercaptoethanol, and 8% glycerol adjusted to pH 7.5 with Tris base. Crude soybean phospholipids were suspended at 40 mg/ml in the same medium without glycerol and sonicated to clarity in a bath sonicator (Laboratory Supplies Co., New York). Equal volumes (0.5-1.5 ml) of enzyme and lipid solutions were mixed in nitrocellulose tubes (Beckman, $\frac{5}{8} \times 4$ inches) and concentrated MgCl_2 were added to a final concentration of 5 mM. The mixture was placed for 1 h in a freezer at -70 °C and thawed in a water bath at room temperature. The turbid suspension was finally clarified by sonication for 3 min at 15 °C.

Assay of $^{32}\text{P}_i$ -ATP Exchange—Fifty μl of reconstituted proteoliposomes were mixed with 100 μl of water and 50 μl of 250 mM Mes and 50 mM MgSO_4 adjusted to pH 5.5 with Tris base. After 2 min of preincubation at 30 °C, the reaction was started with 20 μl of 0.25 M $\text{Tris}/^{32}\text{P}_i$ (about 1,000 cpm/nmol) and 25 μl of 0.1 M Tris/ATP . Following 10 min of incubation at 30 °C, the reaction was stopped with 2 ml of 0.5 N perchloric acid and, after addition of 1 ml of 2.5% ammonium molybdate, the nonesterified phosphate was extracted three times with 3 ml of isobutyl alcohol. Aliquots of 1.5 ml of the aqueous phase were counted by the Cerenkov radiation with an efficiency of about 60%.

Assay of ATPase Activity—The conditions were identical as for the $^{32}\text{P}_i$ -ATP exchange except that the $\text{Tris}/^{32}\text{P}_i$ was omitted. The reaction was stopped after 1 min of incubation at 30 °C with 0.5 ml of a reagent containing 0.35 N sulfuric acid, 0.5% ammonium molybdate, and 0.5% sodium dodecyl sulfate; 5 μl of 10% ascorbic acid were added and the absorbance at 750 nm was determined after 5 min.

Chemicals— $^{32}\text{P}_i$ (carrier-free) was obtained from the Radiochemical Centre (Amersham). In order to reduce the radioactivity not extracted by isobutyl alcohol (pyrophosphate and polyphosphates)

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¹ The abbreviations used are: ATPase, adenosine triphosphatase (EC 3.6.1.3); Tricine, N-tris(hydroxymethyl)methylglycine; Mes, 2-(N-morpholino)ethanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide; Dinoseb, 2,4-dinitro-6-(2-butyl)phenol; Zwittergent TM 314, 3-(tetradecyldimethylammonium)-1-propanesulfonate.

² F. Malpartida and R. Serrano, manuscript submitted for publication.

from the original value of 0.5–0.7% to less than 0.03%, the $^{32}\text{P}_i$ was boiled in 1 N HCl for 4–6 h. Finally, it was diluted to about 1,000 cpm/nmol with phosphoric acid and adjusted to pH 5.5 with Tris base.

Tris/ATP was prepared from Na/ATP (Sigma, grade I) as described (10). Crude soybean phospholipids (Sigma phosphatidylcholine type II-II-S) were washed with acetone (19).

The following products were obtained from Sigma: egg yolk phosphatidylcholine (type III-E), egg yolk phosphatidylethanolamine (type III), pyruvate kinase (type II), hexokinase (type C-300), Mes, diethylstilbestrol, oligomycin, CCCP, valinomycin, gramicidin D, phosphoenolpyruvate (potassium salt), ITP, UTP, GTP, and CTP. Tris and DCCD were obtained from Merck, Tricine from Calbiochem, and Dinoseb from Serva. Zwittergent TM 314 was a gift of A. Seeley (Calbiochem, Luzerne, Switzerland) and nigericin was donated by Dr. W. E. Scott (Hoffman-La Roche, Nutley, NJ).

Crude mitochondrial phospholipids were prepared by extracting yeast mitochondria (9) by the method of Bligh and Dyer as described in Ref. 21. The washed lipid extract was dried, redissolved in ether, and precipitated with 4 volumes of acetone to remove neutral lipids.

RESULTS AND DISCUSSION

Purified yeast plasma membrane ATPase reconstituted with phospholipids by a freeze-thaw-sonication procedure (18) and incubated with $^{32}\text{P}_i$ and ATP catalyzed an esterification of $^{32}\text{P}_i$ which was linear for 10 min. That the compound formed was in fact [γ - ^{32}P]ATP was shown by adsorption on charcoal and hydrolysis in 1 N HCl for 7 min at 100 °C; after treatment with hexokinase (40 units/ml) and glucose for 5 min at pH 8, the esterified radioactivity resisted acid hydrolysis (data not shown). Therefore, the observed reaction corresponded to a $^{32}\text{P}_i$ -ATP exchange. The initial rate of exchange varied for different preparations from 15 to 35 nmol/min \times mg, three orders of magnitude lower than the ATPase activity of the same preparations. When pyruvate kinase (0.3 mg/ml) and phosphoenolpyruvate (10 mM) were included in the assay, the $^{32}\text{P}_i$ -ATP exchange was inhibited by 90%, in accordance with a requirement for ADP coming from ATP hydrolysis.

The ATPase activity of the purified yeast plasma membrane ATPase was only partially dependent on exogenous phospholipids and maximum activity could be obtained by simple mixing of the enzyme with phospholipids during the assay. On the other hand, the $^{32}\text{P}_i$ -ATP exchange was absolutely dependent on the sonication of the enzyme in the presence of phospholipids. Omitting either the phospholipids or the sonication in the reconstitution procedure resulted in undetectable exchange (less than 0.5 nmol/min \times mg). This may indicate that incorporation of the enzyme into the membrane of phospholipid vesicles is essential for the $^{32}\text{P}_i$ -ATP exchange (19, 20). As previously reported for other membrane enzymes (18), freezing of the ATPase with the phospholipids before the sonication greatly improved the reconstitution (about 10-fold). Slow freezing of the samples by placing them in a freezer gave slightly higher exchange than rapid freezing in liquid nitrogen or dry ice-acetone. The inclusion of 5 mM MgCl_2 also improved the reconstitution and this corresponded to a nonspecific ionic effect because similar results were obtained with 100 mM KCl. Crude soybean phospholipids proved to be the best lipids for the reconstitution although acceptable exchange rates (5–10 nmol/min \times mg) could be obtained with crude mitochondrial phospholipids and with mixtures of purified phosphatidylcholine and phosphatidylethanolamine.

That the $^{32}\text{P}_i$ -ATP exchange and the hydrolysis of ATP were catalyzed by the same enzyme was suggested by the following observations: (a) both the ATPase and the exchange activities were very specific for ATP and when this nucleotide was substituted by ITP, UTP, CTP, or GTP both activities were reduced to less than 5%; (b) both reactions exhibited a similar pH dependence, with a maximum rate between pH 5.5 and 6.0 and less than 10% activity at pH 4.0 and 8.0; (c) the

ATPase inhibitors diethylstilbestrol and DCCD produced a similar inhibition of ATP hydrolysis and $^{32}\text{P}_i$ -ATP exchange (Table I). The acidic pH optimum and the lack of sensitivity to the specific mitochondrial ATPase inhibitors (9) oligomycin and DCCD at low concentrations (Table I) indicate that the observed $^{32}\text{P}_i$ -ATP exchange was not caused by contaminating mitochondrial ATPase (19, 20).

The effect of different ionophores (22) on the $^{32}\text{P}_i$ -ATP exchange was studied with Tris as the only added cation and also in the presence of potassium (Fig. 1). In the absence of potassium, the nonspecific ionophore gramicidin produced complete inhibition of the reaction. This indicates that a gradient of some monovalent cation(s) is implicated in the process. As no monovalent cation were added to the assay medium, the participation of an electrochemical proton gradient was the most obvious possibility. However, the proton ionophore (uncoupler) CCCP, even when utilized at high concentration, produced only up to 40% inhibition. Therefore, in addition to the proton, another monovalent cation inadvertently introduced into the system may be transported by the ATPase. Nigericin, which catalyzes the electroneutral exchange of protons for potassium (22), produced up to 60% inhibition of the $^{32}\text{P}_i$ -ATP exchange (Fig. 1) and the potassium ionophore valinomycin, which by itself was ineffective (Fig. 1), increased the inhibitory effect of CCCP to 75% (data not shown). These results clearly implicate the participation of potassium in the transport activity of the enzyme.

Analysis of reaction mixtures by atomic absorption spectrophotometry indicated that they contained from 20–30 μM potassium, probably introduced as a contaminant of the chemicals employed. When the exchange reaction was conducted in the presence of 20 mM KCl, the rate in the absence of ionophores was not affected but the inhibition by nigericin was increased to 75% (Fig. 1) and the combination of uncouplers (CCCP or Dinoseb) with valinomycin produced complete inhibition (Table I). On the other hand, the inhibition by CCCP was slightly reduced in the presence of potassium (Fig. 1).

As indicated in Table I, the effect of the ionophores cannot be explained by a nonspecific inhibition of the ATPase activity. Actually, under conditions of complete inhibition of the $^{32}\text{P}_i$ -ATP exchange (with gramicidin and CCCP plus valinomycin), the ATPase activity was significantly stimulated (up to 30%). This is in accordance with the generation by the reconstituted ATPase of ion gradients dissipated by the ion-

TABLE I
Effect of ATPase inhibitors and ionophores on the $^{32}\text{P}_i$ -ATP exchange and ATPase activities of the reconstituted proteoliposomes

ATPase inhibitors and ionophores were added in 2 μl of methanol to the indicated final concentration and preincubated with the proteoliposomes for 2 min before starting the reaction. The standard incubation medium (see "Materials and Methods") was supplemented with 20 mM KCl.

Additions	$^{32}\text{P}_i$ -ATP ex- change	ATPase
	nmol/min \times mg	$\mu\text{mol/min} \times \text{mg}$
None (2 μl methanol)	24.5	14.5
Oligomycin (80 $\mu\text{g/ml}$)	23.7	14.3
DCCD (16 $\mu\text{g/ml}$)	23.3	11.7
DCCD (120 $\mu\text{g/ml}$)	3.7	4.2
Diethylstilbestrol (120 $\mu\text{g/ml}$)	4.9	2.2
Valinomycin (1.6 $\mu\text{g/ml}$)	22.0	14.4
CCCP (8 $\mu\text{g/ml}$)	17.2	15.3
Dinoseb (13 $\mu\text{g/ml}$)	16.0	
CCCP and valinomycin	<0.5	17.4
Dinoseb and valinomycin	2.4	
Gramicidin (8 $\mu\text{g/ml}$)	<0.5	18.9
Nigericin (4 $\mu\text{g/ml}$)	6.2	16.0

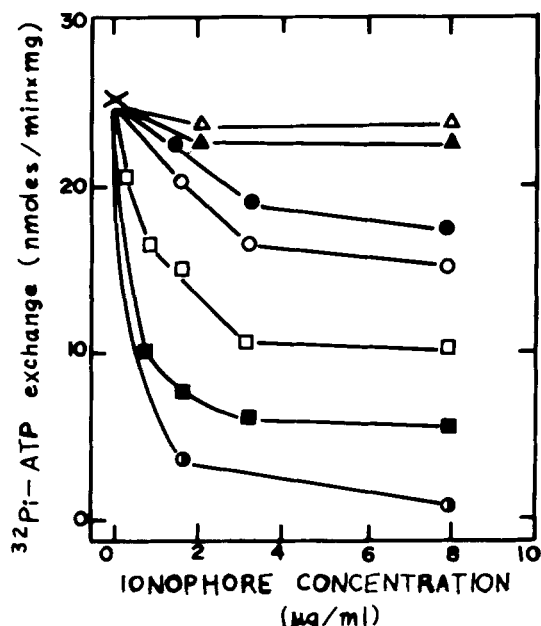


FIG. 1. Inhibition of $^{32}\text{P}_i$ -ATP exchange by ionophores. The ionophores valinomycin (Δ , Δ), CCCP (\circ , \bullet), nigericin (\square , \blacksquare), and gramicidin (\bullet) were added in $2\ \mu\text{l}$ of methanol and preincubated with the proteoliposomes for 2 min before starting the reaction. Open symbols correspond to the standard incubation medium with Tris as the only cation. Closed symbols indicate that 20 mM KCl was included. In the case of gramicidin and of the controls with methanol (\times), the exchange rate was identical with and without KCl.

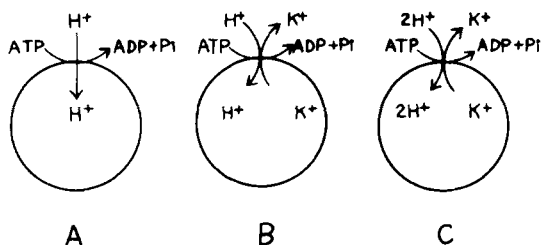


FIG. 2. Scheme of the possible modes of operation of the yeast proton pump in the reconstituted system. A, electrogenic proton pump; B, electroneutral proton-potassium exchange; C, electrogenic proton-potassium exchange.

ophores which drove the exchange and inhibited the ATPase (19).

The pattern of ionophore sensitivity of the $^{32}\text{P}_i$ -ATP exchange is difficult to rationalize in terms of any single mechanism of operation of the yeast plasma membrane ATPase (Fig. 2). The reaction of an electrogenic proton pump (Fig. 2A) should be prevented by uncouplers. On the other hand, the exchange due to an electroneutral proton-potassium exchange (Fig. 2B) should be completely inhibited by nigericin. However, both types of ionophores produced only partial inhibition. An electrogenic proton-potassium exchange (Fig. 2C) would also be incompatible with the experimental results. In the absence of passive permeability to protons or potassium, this type of pump can only generate an electrical potential and therefore the inhibition by nigericin would not be explained. That the vesicles are essentially impermeable to protons and potassium is suggested by the requirement for both proton and potassium ionophores to completely inhibit the $^{32}\text{P}_i$ -ATP exchange.

A plausible explanation for our results is that the purified yeast plasma membrane ATPase utilized for the present experiments is heterogeneous in transport activity, part of the enzyme molecules operating as electrogenic and part as elec-

troneutral pumps. When the potassium concentration is raised, the inhibition by nigericin increases and that by CCCP decreases. This may indicate that the electrogenic component does not require potassium and that the electroneutral exchange was not saturated with the micromolar levels of potassium introduced as a contaminant in the assay medium. The molecular basis for this transport heterogeneity is currently under investigation and it is possible that the minor polypeptides of low molecular weight present in the preparation² (12) may be involved.

This work constitutes the first demonstration of energy transfer activities with purified ATPases from fungal plasma membranes and the sensitivity of the $^{32}\text{P}_i$ -ATP exchange to proton ionophores provides experimental evidence for the contention (13–17) that these enzymes operate as proton pumps. On the other hand, the indication that the purified ATPase mediates potassium transport is in line with recent reports in plants (23, 24) and yeast (25) that potassium uptake cannot be explained on energetic grounds by the membrane potential and therefore must be directly coupled to ATP hydrolysis. The reconstitution procedure developed in the present work would greatly facilitate the study of the physiological function and reaction mechanism of the proton-translocating ATPases of the plasma membranes of fungi, plants, and algae.

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