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Spontaneous Insertion of Plant Plasma Membrane (H^+)ATPase into a Preformed Bilayer

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Summary. The purified (H^+)ATPase from corn roots plasma membrane inserted spontaneously into preformed bilayer from soybean lipids. The yield of the protein insertion, as measured from its H^+-pumping activity, increased as a function of lipids and protein concentrations. In optimum conditions, all the (H^+)ATPase molecules were closely associated with liposomes, exhibiting a high H^+-pumping activity (150,000% quenching · min^{-1} · mg^{-1} protein of the probe 9-amino-6-chloro-2-methoxyacridine). The insertion was achieved within a few seconds. No latency of the (H^+)ATPase hydrolytic activity was revealed when lysophosphatidylcholine was added to permeabilize the vesicles. This indicated that the (H^+)ATPase molecules inserted unidirectionally, the catalytic sites being exposed outside the vesicles ("inside-out" orientation), and thus freely accessible to Mg-ATP. The nondelipidated (H^+)ATPase could also functionally insert into bilayer from PC : PE : PG or PC : PE : PI, due to the presence of both hydrophobic defects promoted by PE, and negative phospholipids specifically required by the (H^+)ATPase from corn roots. The detergent octylglucoside facilitated the delipidated (H^+)ATPase reinsertion probably by promoting both a proper protein conformation and hydrophobic defects in the bilayer. Lysophosphatidylcholine facilitated the delipidated protein insertion only when hydrophobic defects were already present, and thus seemed only capable to ensure a proper protein conformation

Key Words (H^+)ATPase · plant plasma membrane · spontaneous insertion · liposomes · corn root

Introduction

A few membrane proteins have been successfully reconstituted into preformed liposomes by spontaneous insertion: mitochondrial ATPase (Eytan, Schatz & Racker, 1976), cyt b_5 (Enoch, Fleming & Strittmatter, 1979), cyt *c* oxidase (Eytan et al., 1976), UDPglucoronosyltransferase from pig liver microsomes (Scotto & Zakim, 1985), and bacteriorhodopsin (Scotto & Zakim, 1985, 1986; Rigaud, Paternostre & Bluzat, 1988).

The energization of plasma membrane in plant and fungi essentially results from a highly electrogenic H^+-pumping (H^+)ATPase (E.C 3.6.1.35) which constitutes a novel group of ion-pumping enzyme (Serrano, 1989). Nevertheless, similarly to the (Na^+,K^+)-, (H^+,K^+)- and (Ca^{2+})-pumping ATPases identified in animal cells, the ATP hydrolysis proceeds with the formation of a covalent phosphorylated intermediate of 100 kD (Briskin, 1988), and is inhibited by the bivalent form of orthovanadate (Gibrat et al., 1989). The (H^+)ATPase gene has been cloned in yeasts, fungus, and higher plants (Serrano, 1989). Some details of the structure of the enzyme have been deduced from sequence and mutational analysis in yeast. Nine transmembrane helixes are predicted, with the most part of the remaining peptidic chain located at the cytosolic face. Six domains present strong homologies with other ion-transporting ATPases from bacteria and the (Na^+,K^+)-, (H^+,K^+)- and (Ca^{2+})-transporting ATPases from animals.

Since this enzyme exhibits both Mg-ATP hydrolysis and H^+-pumping activities, the analysis of its reconstitution is facilitated. In this paper, we show for the first time that the (H^+)ATPase purified from a plant plasma membrane reinserted spontaneously into a preformed bilayer.

Materials and Methods

Plant Material

Corn seeds (*Zea mays* L., var Mona) were surface sterilized for 15 min with calcium hypochlorite, rinsed in running tap water, and grown hydroponically for five days in the dark on a plastic grid over an aerated solution containing 0.1 mM $CaSO_4$. The solution was changed once.

Preparation of Plasma Membrane Vesicles and (H^+)ATPase

Corn root plasma membrane vesicles were purified by phase partition according to the batch procedure of Widell, Lundberg and Larsson (1982), as described by Grouzis et al. (1990). Semi-purified (H^+)ATPase was solubilized by lysophosphatidylcholine (lysoPC), delipidated by cholate treatment, and precipitated in ammonium sulphate, as described by St. Marty-Fleurence et al. (1988). The activity, in the presence of soybean lipids, was routinely 1 μmol · min^{-1} · mg^{-1} protein. (H^+)ATPase was purified, after lysoPC solubilization, on a linear glycerol gradient according to Grouzis et al. (1990). The two-dimensional gels of the purified fraction revealed only the two typical associated spots in the 100-kD region corresponding to isoelectric pH of approximately 6.5. The activity was routinely 8 to 10 μmol · min^{-1} · mg^{-1} protein.

Preparation of Liposomes

Soybean phospholipids (100 mg L-α-phosphatidylcholine, type II-S, Sigma) were dispersed by vigorous mixing on a Vortex mixer in the presence of glass beads, for 15 min under argon, in 1 ml 10 mM Tris/MES buffer, pH 6.5, containing 200 mM (Figs. 1–4) or 100 mM KCl (Tables 1–3). Pure lipids (PC, PE, PI, PG and cholesterol) dissolved in chloroform were dried down in a round flask under a stream of argon, and resuspended by gentle shaking in the buffer described above. Afterwards, soybean lipids or pure phospholipids were clarified by sonication for 15 min under argon in a Bransonic bath sonicator. Insertion of (H^+)ATPase was achieved by mixing aliquots of liposomes and protein on a Vortex mixer as indicated in the Results.

Proton Transport Activity

The initial rate of fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) was used to monitor the H^+-pumping activity of reconstituted vesicles. Fluorescence was measured with a Jobin-Yvon JY3-CS spectrofluorometer fitted out with a stirred and thermostated cell, at excitation/emission wavelengths of 420/485 nm. The standard assay medium (2 ml) contained 15 mM BTP/Cl (pH 6.5), 200 mM KCl, 0.25 μM valinomycin, 3 mM ATP/BTP (pH 6.5), 1 μM ACMA and proteoliposomes (0.5 μg protein, unless otherwise indicated). The presence of valinomycin was required to short circuit the electrogenic (H^+)ATPase, which allows maximum H^+-pumping activity (Gibrat et al., 1990). The reaction at 30°C was started by addition of $MgCl_2$ (3 mM final concentration). The initial rate of quenching was linear with protein concentration and thus could be expressed in specific units (% quenching · min^{-1} · mg^{-1} protein).

ATP Hydrolysis Activity

The activity of (H^+)ATPase was determined by measuring the release of P_i according to the method of Ames (1966). SDS (0.75%, wt/vol) was added to solubilize liposomes. The standard incubation medium (0.5 ml final volume) contained, unless otherwise indicated, 35 mM BTP/Cl (pH 6.5), 50 mM KCl, 3 mM ATP/BTP (pH 6.5), and 3 mM $MgCl_2$. The reaction was started by addition of proteoliposomes or (H^+)ATPase (0.3 μg protein) and was allowed to proceed for 30 min at 30°C.

For lipid reactivation study of the semipurified and delipidated (H^+)ATPase, protein and sonicated lipids were added at a concentration of 10 μg · ml^{-1} and 1.25 mg · ml^{-1}, respectively, and (H^+)ATPase was assayed as described above.

Other Methods

Protein content was assayed by the method of Schaffner and Weissman (1973). Lipid phosphorus was measured as in Gomori (1942).

Source of Chemicals

ATP was obtained from Boehringer Mannheim as disodium salt and converted to BTP salt by filtration through Dowex 50W. ACMA (9-amino-6-chloro-2-methoxyacridine) was obtained from Molecular Probes, PEG (polyethylene glycol) was from Union Carbide, Dextran T500 from Pharmacia. BTP (*bis*-Tris propane), soybean phospholipids (phosphatidylcholine, type II-S), lysoPC (lysophosphatidylcholine, from egg yolk), PC (phosphatidylcholine, from egg yolk), PE (phosphatidylethanolamine, from soybean), PI (phosphatidylinositol, from soybean), PG (phosphatidylglycerol, from egg yolk), cholesterol, CHAPS (3-[(cholamidopropyl)dimethylammonio] 1-propanesulfonate) were from Sigma. Cholate was from Merck. Deoxycholate was from Aldrich. OG (octylglucoside) and all others chemicals were purchased from Boehringer Mannheim.

Results

Effect of the Concentration of Preformed Liposomes from Soybean Lipids on the Spontaneous Insertion of Purified (H^+)ATPase

Twenty μl of purified (H^+)ATPase (20 μg/ml) were added to an aliquot of liposomes from soybean lipids (type II S, Sigma) on a Vortex mixer and vigorously mixed for 5 sec. Diluted (1 ml of 2 mg/ml), or concentrated (20 μl of 100 mg/ml) aliquots of liposomes were used, at this mixing step, such as the concentration of lipids was respectively 2 or 50 mg/ml, whereas the lipid-to-protein ratio was 5,000 (wt/wt). The lipid-protein mixture was then transferred immediately to the cuvette containing the H^+-pumping assay medium supplemented with $MgCl_2$. It is important to note that, in both cases, the final concentrations of lipids (1 mg/ml) and protein (0.2 μg/ml) were the same in the assay medium and only varied at the mixing step.

When diluted aliquots of liposomes were used, a slow decrease of the ACMA fluorescence was observed, denoting a low H^+-pumping activity (Fig. 1, curve *A*). The specific initial rate of quenching (V_{H^+}) was 1,250% quenching · min^{-1} · mg^{-1} protein. When concentrated liposomes were used, a rapid fluorescence quenching was observed (Fig. 1, curve *B*). The V_{H^+} value was 80-fold higher than that previously ob-

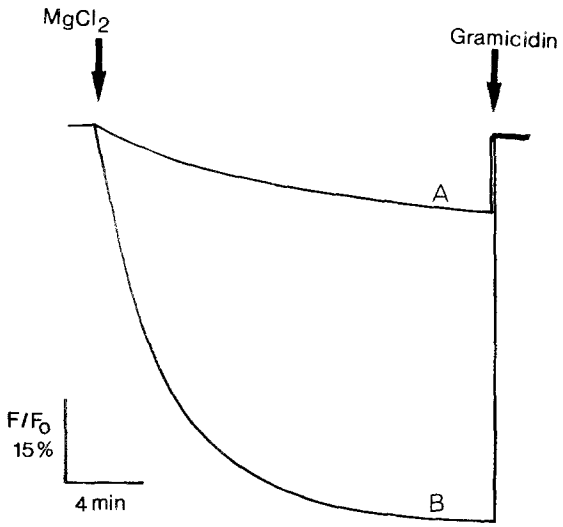

Fig. 1. H^+-pumping activity of the (H^+)ATPase after spontaneous insertion into liposomes of lipids. Traces are the quenching of ACMA fluorescence (F/F_o). For the two curves (*A* and *B*), spontaneous insertion of the purified (H^+)ATPase was achieved by mixing protein and liposomes under vortex for 5 sec (*see* text). Lipid and protein concentrations at the mixing step were, respectively, 2 mg/ml and 0.4 μg/ml (curve *A*), or 50 mg/ml and 10 μg/ml (curve *B*). Proteoliposomes were immediately transferred to the quenching assay medium. The final protein and lipid concentrations in the assay cuvette were, respectively, 0.2 μg/ml and 1 mg/ml, for both curves *A* and *B*. The reaction was started, 1 min later, with a concentrated aliquot of $MgCl_2$ (3 mM final)

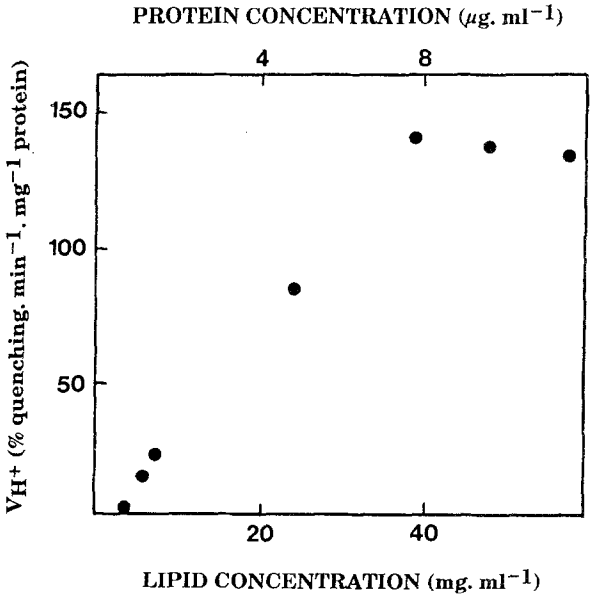

Fig. 2. Dependence of the (H^+)ATPase spontaneous insertion on the lipids and protein concentrations, as measured from its H^+-pumping activity. The protein and lipid concentrations only varied at the mixing step (*see* text), but were constant in the assay medium (0.2 μg/ml and 1 mg/ml, respectively). The values of V_{H^+} are divided by 10^3

served. In both cases, the quenching of the dye could be totally reversed by addition of gramicidin.

The V_{H^+} value was measured as a function of lipids and protein concentrations by the same protocol. The lipid-to-protein ratio was maintained constant at 5,000 (wt/wt); lipids and protein concentrations varied only at the mixing step. The V_{H^+} value increased regularly as a function of lipids and protein concentrations and reached a plateau at approximately 40 mg/ml lipids and 8 μg/ml protein (Fig. 2). At the optimum lipids and protein concentrations, the maximum H^+-pumping activity was already reached after 15 sec, the shortest time we have tested (*data not shown*).

Accessibility of Mg-ATP to the Catalytic Site after Spontaneous Insertion of the Purified (H^+)ATPase into Liposomes from Soybean Lipids

The mean hydrolytic activity of the purified (H^+)ATPase was 8.5 μmol $P_i \cdot min^{-1} \cdot mg^{-1}$ protein. The hydrolytic activity was not significantly different after spontaneous insertion of the enzyme into liposomes (Fig. 3). Addition of the permeabilizing agent lysoPC to the H^+-pumping assay medium caused a decrease of V_{H^+}, which vanished above 1

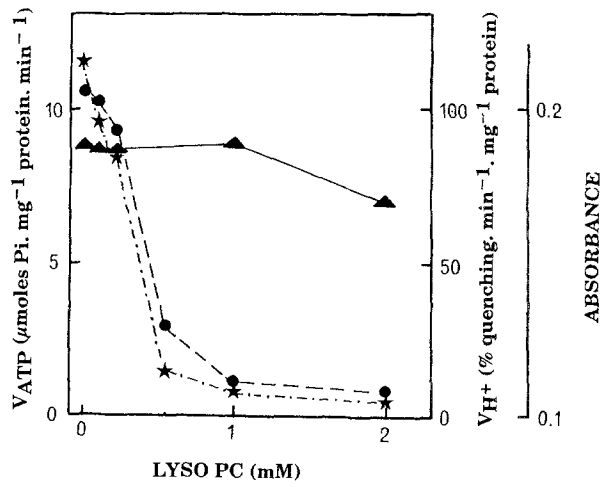

Fig. 3. Effect of lysoPC on the (H^+)ATPase activity and on turbidity of the vesicle suspension after spontaneous insertion. Spontaneous insertion was achieved by mixing liposomes and protein (50 mg/ml and 10 μg/ml, respectively). Thereafter, proteoliposomes were diluted for H^+ pumping (●) and MgATP hydrolysis (▲) activities assays in the presence of increasing concentrations of lysoPC. Turbidity (★) of the suspension (2 mg/ml lipids) was measured at 400 nm. The values of V_{H^+} are divided by 10^3

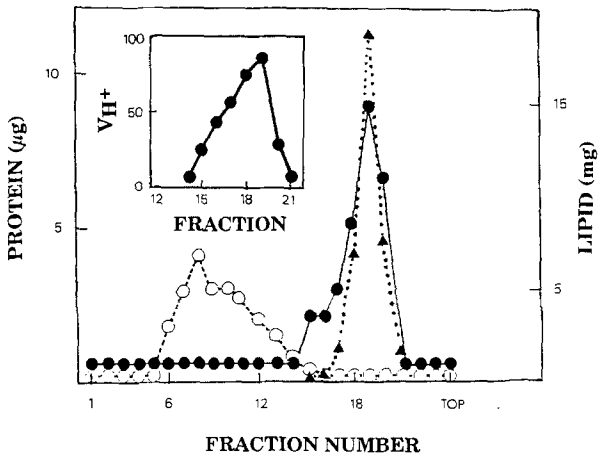

Fig. 4. Distribution of (H^+)ATPase after centrifugation into a linear glycerol gradient, before and after spontaneous insertion into preformed liposomes. (○) control (25 μg of purified (H^+)ATPase); (●) 25 μg of purified (H^+)ATPase (10 μg/ml) were mixed with 125 mg liposomes (50 mg/ml). The sample was applied to a linear glycerol gradient (34 ml, 25 to 50% vol/vol, in 1 mM Tris/Mes, pH 6.5, 1 mM DTT), centrifuged for 16 hr at 150,000 × *g*, and fractions (1.5 ml) were assayed for lipids (▲), protein (●), and H^+-pumping activity (*inset*). The values of V_{H^+} are divided by 10^3

mM (Fig. 3). Upon addition of lysoPC in the same concentration range, a strong decrease of the turbidity of the suspension was observed at 400 nm, due to the solubilization of proteoliposomes. Nevertheless, the Mg-ATP hydrolysis was not affected by lysoPC up to 1 mM.

Distribution of Soybean Lipids and Purified (H^+)ATPase after Centrifugation into a Glycerol Gradient

After centrifugation in a glycerol density gradient, the profiles of the distribution of purified (H^+)ATPase and soybean lipids are shown in Fig. 4. The purified (H^+)ATPase, in the absence of liposomes, was totally recovered in fractions 6–13. After (H^+)ATPase insertion, the protein and the liposomes comigrated in fractions 15–22. The protein and lipids initially layered were totally recovered in these fractions. Proton transport activity was shown in the same fractions (Fig. 4, *inset*).

Spontaneous Insertion of the Purified (H^+)ATPase into Preformed Liposomes with Different Lipid Composition

The effect of the lipid composition of the preformed liposomes on the functional insertion of the purified (H^+)ATPase, as measured from the H^+-pumping activity, is shown in Table 1. Mixing of the (H^+)ATPase and liposomes from pure PC, or from different mixtures of PC : PE, PC : PG, PC : PE : cholesterol, in the optimum conditions previously used with soybean lipids, were uncapable to regenerate the H^+-pumping activity. High H^+-pumping activities, 60 to 90% of that observed with soybean lipids, were recovered when mixtures of PC : PE were supplemented with PI or PG. Further addition of cholesterol slightly increased the activity.

Table 1. Effect of the lipidic composition on the spontaneous insertion of the (H^+)ATPase into a preformed bilayer[a]

Lipids	Ratio (wt/wt)	Relative H^+-pumping activity (%)
Soybean lipids		100
PC		0
PC : PE	2 : 1	0
PC : PE	1 : 2	0
PC : PG	6 : 1	0
PC : PE : PI	5 : 4 : 1	86
PC : PE : PG	3 : 3 : 1	62
PC : PE : Chol[b]	8 : 8 : 1	0
PC : PE : PG : Chol[b]	8 : 8 : 3 : 1	71

[a] The spontaneous insertion of the purified (H^+)ATPase was achieved according to the protocol described in Fig. 1, at the maximum lipid and protein concentrations (40 mg/ml and 8 μg/ml, respectively) determined in Fig. 2.
[b] Cholesterol.

The phospholipid requirement of the (H^+)ATPase hydrolysis activity was investigated using the delipidated (inactivated) semipurified protein preparation. Mixing this protein and different lipids used in Table 1 failed to regenerate H^+-pumping activity (*data not shown*). Only the ATP hydrolysis activity could be regenerated (Table 2). Optimal activation of the semipurified (H^+)ATPase was obtained by soybean lipids. LysoPC was less effective (30% of the activity obtained with soybean lipids). PC, PC : PE and PC : PE : cholesterol were ineffective. The addition of PG into PC, PC : PE and PC : PE : cholesterol mixtures produced an activation of the (H^+)ATPase hydrolytic activity (70, 57 and 50%, respectively).

Effect of Different Detergents on the Spontaneous Insertion of the Semipurified and Delipidated (H^+)ATPase into Preformed Liposomes from Soybean Lipids

In contrast to the nondelipidated (purified) (H^+)ATPase, the delipidated (semipurified) (H^+)ATPase could not spontaneously reinsert in li-

Table 2. Effect of different lipids on the activation of the delipidated semipurified (H⁺)ATPase[a]

Lipids	Ratio (wt/wt)	Relative ATPase hydrolysis activity (%)
None		0
Soybean lipids		100
PC		12
PC : PE	2 : 1	0
PC : PG	6 : 1	73
PC : PI	6 : 1	94
PC : PE : PG	3 : 3 : 1	57
PC : PE : Chol[b]	8 : 8 : 1	0
PC : PE : PG : Chol[b]	8 : 8 : 3 : 1	50
LysoPC		22

[a] The enzyme was mixed with the lipids before starting the reaction as described in Materials and Methods.
[b] Cholesterol.

Table 3. Effect of different detergents on the spontaneous insertion of the delipidated semipurified (H⁺)ATPase into a preformed bilayer[a]

Detergent	concentration (mM)	Lipid	Relative H⁺-pumping activity (%)
OG:	26.0 (4.0)	Soybean lipids	100
	26.0 (4.0)	PC : PE : PG (3 : 3 : 1)	100
	26.0 (4.0)	PC : PG (17 : 3)	43
	26.0 (4.0)	PC	0
LysoPC:	7.0 (1.4)	Soybean lipids	100
	1.5 (0.3)	Soybean lipids	75
	7.0 (1.4)	PC : PE : PG (3 : 3 : 1)	60
	7.0 (1.4)	PC : PG (17 : 3)	0
Cholate:	27.0 (4.0)	Soybean lipids	0
	21.0 (3.6)	Soybean lipids	0
	15.0 (2.5)	Soybean lipids	0
DOC:	7.0 (1.0)	Soybean lipids	0
	8.5 (1.3)	Soybean lipids	0
	10.0 (1.6)	Soybean lipids	0
CHAPS:	12.0 (1.7)	Soybean lipids	0
	18.0 (2.5)	Soybean lipids	0

[a] OG: octylglucoside; DOC: deoxycholate; CHAPS: 3-(3-cholamidopropyl-dimethylammonio)-1-propane sulfonate. The indicated concentrations refer to the detergent concentrations in the presence of the protein before mixing with lipids, and the values in parentheses refer to the concentration after addition of lipids. The mixture was added to the H⁺-pumping assay medium, as described in Materials and Methods.

posomes of the different lipid composition used. We studied the effect of different detergents on the spontaneous insertion of the delipidated (H⁺)ATPase, by supplementing the protein with detergents, before mixing with liposomes of different lipid compositions. We verified that the turbidity of the liposome suspension did not decrease upon detergent-protein mixture addition (*data not shown*).

When octylglucoside was added to delipidated protein, before mixing with liposomes from soybean lipids, H^+ pumping could be regenerated provided that the detergent concentration was above the critical micellar concentration (CMC) (*data not shown*). Proton pumping activity could be also regenerated with octylglucoside, above the CMC, with liposomes from PC : PE : PG or PC : PG mixture, but not from pure PC (Table 3). LysoPC was as effective as octylglucoside when liposomes from soybean lipids or from PC : PE : PG were used, but not with liposomes from PC : PG mixture. The zwittergent CHAPS, the anionic detergents DOC and cholate could not regenerate the H^+-pumping activity with liposomes from soybean lipids, whatever the concentration used.

Discussion

Spontaneous Insertion of the (H⁺)ATPase into Liposomes of Soybean Lipids

The value of V_{H^+} increased as a function of lipids and protein concentrations at the mixing step (Fig. 2). This suggests that the probability of the insertion depends on the frequency of collisions between (H⁺)ATPase molecules and liposomes. In the optimum condition, all the (H⁺)ATPase molecules were closely associated with lipids and competent for H^+ pumping (Fig. 4).

At the optimum lipids and protein concentrations, the spontaneous insertion of the (H⁺)ATPase was achieved within a few seconds (*data not shown*). This is in agreement with the reported times (from a few seconds to a few minutes) for the spontaneous insertion of the H^+-pumping proteins bacteriorhodopsin and cyt *c* oxidase (Eytan et al., 1976; Scotto & Zakim 1985; Rigaud et al., 1988).

The high hydrolytic activity of the purified (H⁺)ATPase (8.5 μmol $P_i \cdot min^{-1} \cdot mg^{-1}$ protein) was not affected after spontaneous insertion of the protein into liposomes (Fig. 3). This suggests that all the catalytic sites of the reconstituted ATPase molecules were exposed to the outside of the vesicles, and thus remained freely accessible to Mg-ATP.

Low concentrations (close to the critical micellar concentration) of lysoPC, or other surfactants such as Triton X-100 or SDS, have been used as permeabilizing agents to reveal the latent (H⁺)ATPase hydrolytic activity of right-side out vesicles in

native plasmalemma preparations (Grouzis et al., 1987; Galtier et al., 1988; Palmgren & Sommarin, 1989; Monk et al., 1990). In the present reconstituted preparation, the large decrease of the H^+-pumping activity was caused by the solubilizing effect of lysoPC, shown by the large decrease of the turbidity (Fig. 3). Nevertheless, lysoPC did not stimulate the hydrolytic activity. This absence of latent (H^+)ATPase hydrolytic activity confirms that all the catalytic sites of the enzyme were exposed to the outside of the vesicles after spontaneous insertion.

Most of the procedures developed for membrane protein reconstitution made use of a detergent (Racker et al., 1979). Generally, a scrambling of the protein orientation is observed with such procedures (Eytan, 1982; Helenius, Sarvas & Simons, 1981). Conversely, mitochondrial ATPase, cyt *c* oxidase (Eytan et al., 1976), and bacteriorhodopsin (Rigaud et al., 1988) were shown to be unidirectionally reincorporated after spontaneous insertion. Our present study shows that the plant plasma (H^+)ATPase also inserts unidirectionally into a preformed bilayer.

Spontaneous Insertion of (H^+)ATPase into a Preformed Bilayer of Different Lipid Mixtures

The assumption generally made to explain the spontaneous insertion of membrane proteins is the presence of organizational defects in the phospholipid bilayer (Jain & Zakim, 1987). Striking similarities in the characteristics of direct incorporation of membrane proteins and membrane–membrane fusion have already been pointed out (Eytan, 1982; Scotto & Zakim, 1985). The membranes formed from pure PC, or PC : PG (which are cylindrically shaped phospholipids), are known to be regular liquid crystalline (L-α)bilayers, which do not exhibit the defects involved in membrane fusion (Cullis et al., 1985; Chernomordik, Melikyan & Chimadzhev, 1987). Nevertheless, the light-driven (H^+)pump bacteriorhodopsin (Scotto & Zakim, 1985, 1986; Rigaud et al., 1988) spontaneously inserted into phosphatidylcholine bilayer, due to the defects induced by very low amounts of detergents (Scotto & Zakim, 1985; Jain & Zakim, 1987; Rigaud et al., 1988), or fusogens (Scotto & Zakim, 1985).

In the present study, the (H^+)ATPase remained fully active after purification on a glycerol gradient. Probably the purified enzyme remained associated with lipids (mainly lysoPC used for solubilization), since its activity was totally lost after delipidation by cholate treatment (Table 2). Nevertheless, no H^+-pumping activity was obtained after mixing (H^+)ATPase purified on glycerol gradient and liposomes from pure PC (Table 1). This suggests that, if defects were induced by the residual lysoPC, they should not be competent for (H^+)ATPase insertion. Indeed, the defects induced by lysoPC, which is an inverted conically shaped lipid, do not expose locally the hydrophobic interior of the bilayer, but on the contrary lead to the formation of hydrophilic pores (Chernomordik et al., 1987). This should be at the origin of the antifusogenic property of this lipid.

On the other hand, the so-called bulging defects induced by conically shaped lipids, such as PE, give locally access to the hydrophobic interior of the membrane. These defects should be a prerequisite for reaching the prefusional state in membrane-membrane interaction (Chernomordik et al., 1987). Natural lipid mixtures, i.e., asolectin, also contain a high proportion of conically shaped molecules, which should explain the spontaneous fusion of two macroscopic bilayers (Chernomordik et al., 1987). Nevertheless, no reactivation of the H^+-pumping activity of the purified (H^+)ATPase was obtained with PC : PE mixtures (Table 1). Such a functional reinsertion was only observed when mixtures of PC : PE were supplemented with a negatively charged phospholipid (PI or PG). The (H^+)ATPase insertion is not likely to be facilitated by PI or PG *via* defect formation, since these phospholipids are also cylindrically shaped (Cullis et al., 1985).

The hydrolytic activity of the delipidated (inactivated) ATPase can be regenerated only by lipid mixtures containing negatively charged phospholipids (soybean lipids, and PG or PI mixtures) (Table 2). Neutral PC, PE and cholesterol mixtures gave no, or very low, reactivation of the enzyme. Whatever the lipid compositions used, the H^+ pumping could not be regenerated after mixing liposomes and delipidated, aggregated, (H^+)ATPase. In contrast to the nondelipidated (H^+)ATPase (Fig. 4), the delipidated (H^+)ATPase could be separated from the bulk of lipids, after mixing by a centrifugation on a density gradient (*data not shown*). Only a minor fraction of lipids remained associated to the protein, in the bottom of the gradient. This suggests that liposomes were destabilized after mixing with protein aggregates, producing high density lipid-protein micelles. Finally, the lipid selectivity observed in Table 2 seems to result merely from a specific requirement of corn roots (H^+)ATPase for negatively charged phospholipids. Such negative lipid requirement was observed for plasma membrane (H^+)ATPase from *Neurospora crassa* (Scarborough, 1977) and yeast (Serrano, Montesinos & Sanchez, 1988). On the contrary plasmalemma (H^+)ATPase from oat roots can be reactived either by neutral phospholipids (PC, PE), or negatively charged phospholipids (PI, PG, PA, PS) (Serrano et al., 1988). In animal field,

(Na^+,K^+)ATPase requires negatively charged phospholipids (Kimelberg & Papahadjopoulos, 1972), whereas Ca^{2+}-ATPase can be activated by all phospholipids species (Bennet et al., 1976). The lipid requirement of membrane-bound intrinsic enzyme seems not related to tight interaction of proteins and phospholipids (Devaux & Seigneuret, 1985). Its significance, as well as its relation with the available knowledge of the molecular structure of the ATPase, remains to be elucidated.

In conclusion, functional reinsertion of purified and nondelipidated (H^+)ATPase, as demonstrated by the regeneration of the H^+-pumping activity, seems to depend on the presence of both hydrophobic defects, induced by conically shaped phospholipids, and negatively charged lipids (PG, PI), specifically required by the corn roots (H^+)ATPase.

Effect of Detergents on the Spontaneous Insertion of the Delipidated (H^+)ATPase into Preformed Liposomes of Different Lipid Composition

The proton pumping activity could not be regenerated after mixing the delipidated, aggregated, (H^+)ATPase and the different liposomes used in this study. Octylglucoside and lysoPC, but not CHAPS, cholate and deoxycholate, could regenerate the H^+pumping activity when added to the protein aggregates before mixing with liposomes from soybean lipids (Table 3). The amounts of detergents were insufficient to destroy the bilayer. On the other hand, octylglucoside and lysoPC, but not CHAPS, cholate and deoxycholate, are known to solubilize efficiently the (H^+)ATPase from corn roots plasmalemma (Serrano, 1985). Thus, the detergents which are capable to extract the protein from the bilayer also favor the spontaneous reinsertion of the delipidated (H^+)ATPase. These detergents might affect the conformation of the protein and also might induce defects in the bilayer (Jain & Zakim, 1987).

This important point may be appreciated from the comparison of the effect of octylglucoside and lysoPC on the spontaneous insertion of the delipidated (H^+)ATPase into liposomes from different mixtures of pure phospholipids. Firstly, octylglucoside, but not lysoPC, allows the functional reinsertion of the (H^+)ATPase into regular (L-α)bilayer from PC : PG (Table 3). This suggests that only octylglucoside is able to induce hydrophobic defects in such bilayers. Secondly, both of these detergents facilitate the spontaneous insertion of the delipidated protein into bilayer from PC : PE : PG, known to present "built in" hydrophobic defects due to the conically shaped PE. Thus it seems that both octylglucoside and lysoPC favor a protein conformation suitable for its spontaneous insertion when conditions for membrane fusion are met. Finally, the inefficiency of octylglucoside to functionally reinsert the delipidated protein into bilayer from pure PC should be related to the above discussed negative lipid requirement of the (H^+)ATPase.

Conclusions

This paper demonstrates for the first time the functional spontaneous insertion of a plant plasma membrane (H^+)ATPase into a preformed bilayer.

This (H^+)ATPase may reinsert spontaneously into a preformed bilayer, provided that: (*i*) a proper conformation of the protein is maintained, (e.g., with lysoPC or octylglucoside); (*ii*) the bilayer presents hydrophobic defects, promoted by PE, or by octylglucoside; and (*iii*) negatively charged lipids (PG, PI) are present. Soybean lipids, which composition can be roughly compared to that of the native membrane of corn roots (Gronewald et al., 1982), satisfy the two latter conditions. It has been proposed that spontaneous insertion may occur *in vivo* (Jain & Zakim, 1987). This proposal seems especially sound for the assembly into membrane of (H^+)ATPase, which is predicted to loop nine times across the membrane, from the sequencing of the genes of fungi, yeast and higher plants (Serrano, 1989). Finally, the spontaneous insertion of (H^+)ATPase may be a useful tool for the study of the functioning and regulation of ion transport in plant because it allows high-yield reconstitution, with controlled orientation, while preserving the integrity of the preformed acceptor membrane.

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