Plant Physiol. (1989) 90, 1524-1531
0032-0889/89/90/1524/08/$01.00/0

Received for publication January 17, 1989
and in revised form March 27, 1989

Selective Delipidation of the Plasma Membrane by Surfactants[1]

Enrichment of Sterols and Activation of ATPase

Richard P. Sandstrom and Robert E. Cleland*

Department of Biology, Portland State University, Portland, Oregon 97207 (R.P.S.) *and University of Washington, Department of Botany, KB-15, Seattle, Washington 98195* (R.E.C.)

ABSTRACT

The influence of plasma membrane lipid components on the activity of the H^+-ATPase has been studied by determining the effect of surfactants on membrane lipids and ATPase activity of oat (*Avena sativa* L.) root plasma membrane vesicles purified by a two-phase partitioning procedure. Triton X-100, at 25 to 1 (weight/weight) Triton to plasma membrane protein, an amount that causes maximal activation of the ATPase in the ATPase assay, extracted 59% of the membrane protein but did not solubilize the bulk of the ATPase. The Triton-insoluble proteins had associated with them, on a micromole per milligram protein basis, only 14% as much phospholipid, but 38% of the glycolipids and sterols, as compared with the native membranes. The Triton insoluble ATPase could still be activated by Triton X-100. When solubilized by lysolecithin, there were still sterols associated with the ATPase fraction. Free sterols were found associated with the ATPase in the same relative proportions, whether treated with surfactants or not. We suggest that surfactants activate the ATPase by altering the hydrophobic environment around the enzyme. We propose that sterols, through their interaction with the ATPase, may be essential for ATPase activity.

The factors that influence the activity of the PM[2] H^+-ATPase have attracted considerable attention because of the importance of this enzyme in solute uptake, membrane potential and plant growth mechanisms (35). The ATPase exists *in situ* in a lipid environment consisting of phospholipids, sterols, and glycolipids (8, 9, 21, 25, 31). It has been assumed that a specific phospholipid environment is required for optimal activity. For example, treatment of membranes with surfactants, which should cause partial delipidation, generally reduces ATPase activity, while readdition of phospholipid mixtures partially restores activity (8, 11, 19, 34). The amount of ATPase activation in these studies depends on which phospholipids are present.

The possible role of sterols and glycolipids in the activity of plant ATPases has received less attention. It is not known whether surfactants remove sterols during solubilization or activation of ATPases. Sterols are rarely added to the phospholipid mixtures during reconstitution procedures, and the possibility that soluble ATPase brings along sterols does not seem to have been considered.

Surfactants activate the ATPase activity in plasma membrane vesicles that have been isolated by the two-phase partitioning system (3). This is usually explained as a surfactant-induced leakage of ATP into the vesicles, permitting the ATP to reach the active site of the ATPase in right-side out vesicles (22). We have suggested from electron spin resonance data, on the other hand, that Triton X-100 directly activates the ATPase (33) in addition to changing the permeability of vesicles. Triton X-100 might do so by altering the spectrum of lipids surrounding the ATPase.

The present investigation was undertaken to determine the effect of surfactant treatment on the lipid components associated with the plasma membrane ATPase of oat roots. It will be shown that Triton X-100 at 25 to 1 (w/w, Triton to protein), a concentration that causes maximal activation of the ATPase, removes the majority of the phospholipids but leaves considerable glycolipids and sterols associated with the active ATPase preparation.

MATERIALS AND METHODS

Chemicals

Sodium-ATP, dextran (mol wt 500,000), polyethylene glycol (mol wt 3,350), 6-*p*-toluidino-2-naphthalenesulfonic acid, Triton X-100, lysophosphatidyl choline, sodium deoxycholate, and lipid standards were purchased from Sigma (St. Louis, MO). Plant sterol standards were purchased from Supelco (Bellefonte, PA). Octyl-β-D-glucopyranoside was purchased from Calbiochem (San Diego, CA). Solvents were redistilled in glass and used fresh. All other chemicals were reagent grade.

[1] Supported by the National Science Foundation, grant DMB-8502021, and the U.S. Department of Energy, contract DE-AT06-76ER73019.

[2] Abbreviations: PM, plasma membrane; LPC, lysophosphatidylcholine; LPCS, LPC supernatant; HPTLC, high performance TLC; TXP, pellet from Triton X-100 washed PM vesicles; LPCP, pellet from LPC-treated TXP; LPCSF, supernatant from LPC-treated TXP that was filtered through a membrane with a 100,000 mol wt exclusion limit.

Plant Material

Oat (*Avena sativa* L. cv Victory) seeds were germinated and grown on cheesecloth covering a stainless steel screen over a 1 mM $CaSO_4$ solution for 5 d in continuous (dim) red light at 25°C. Roots were harvested at a length of 8 to 10 cm (33).

Extraction of Membrane Vesicles

Two hundred g of roots were ground in a Waring blender containing 400 mL of a buffer solution containing 10 mM Tris/HCl (pH 7.5), 250 mM sucrose, and 1 mM EDTA. The homogenate was filtered through four layers of cheesecloth and centrifuged for 20 min at 10,000 *g* in a fixed angle rotor (r_{max} = 10.5 cm). A microsomal pellet was obtained by centrifuging the supernatant from the first spin for 20 min at 100,000 *g* (33).

Aqueous Phase Partitioning

The microsomal pellet from above was suspended in a buffer solution that contained 5 mM potassium phosphate (pH 7.8), 250 mM sucrose, and 4 mM KCl. The PM vesicles were separated from the microsomal pellet by phase partitioning (22, 33). Briefly, the suspended microsomal pellet was placed in the first tube of a successive, three-tube phase partitioning system where, after addition of vesicles, each tube contained 6.5% (w/w) dextran (500,000 mol wt), 6.5% (w/w) polyethylene glycol (3,350 mol wt), 250 mM sucrose, 5 mM potassium phosphate (pH 7.8), and 4 mM KCl. After shaking the contents of the first tube and centrifuging, the upper phase was transferred to the second tube for partitioning. The procedure was repeated three times. Pure PM vesicles were obtained from the upper phase of the third tube (U_3) by diluting the material in buffer solution containing 10 mM Tris/HCl (pH 7.5), and 250 mM sucrose, centrifuging for 30 min at 100,000 *g*, and suspending the pellet in a smaller volume of the same buffer. The entire procedure was carried out at 4°C.

Triton X-100 and LPC Surfactant Extraction of PM Lipids and Proteins

The initial extraction of U_3 PM lipids and proteins involved the addition of 2 to 4 mg of U_3 PM to Triton X-100 (at a ratio of 25 to 1 Triton to U_3 protein) in 5 to 10 ml of ATPase assay buffer, buffer A: 5 mM $MgSO_4$, 50 mM KCl, 125 mM sucrose, 5 mM Na-ATP (pH 6.5), 0.1 mM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, and 30 mM Mes/Tris (pH 6.5) (33). Five-hundred mM KCl and 125 mM sucrose in 0.3 mM Mes/Tris (pH 6.5) also was found to work. The PM vesicles in the Triton solution were vortexed for 1 min at room temperature in a closed 10 mL polycarbonate centrifuge tube and then centrifuged in a fixed angle rotor (Beckman 50 Ti, r_{max} = 8.1 cm) at 100,000 *g* for 20 min. After removing and saving the supernatant for further analyses, the pellet was suspended in the ATPase buffer minus Triton X-100, vortexed for 1 min at room temperature, and centrifuged as above. This wash procedure was repeated twice and the individual supernatants were saved for further analyses. After the second wash, the pellet, TXP, was again suspended in ATPase assay buffer and sonicated to near clarity. When left on ice for a prolonged period (greater than 1 h), the material in solution began to settle and had to be thoroughly mixed before using. Protein and ATPase analyses were performed on the TXP and the supernatants. LPC was used to further extract the TXP.

The TXP was suspended in a volume equal to the starting volume of ATPase buffer, but containing 0.1% (w/v) LPC, vortexed for 1 min, centrifuged as above, and separated into supernatant (LPCS) and pellet (LPCP) fractions. Two mL of the supernatant were diluted to 50 mL with a Mes/Tris buffer (pH 6.5) containing 500 mM KCl and 125 mM sucrose, and filtered through an Amicon XM-100 membrane filter with a 100,000 mol wt exclusion limit. When the solution was reduced to approximately 2 mL on the filter, it was diluted with an additional 50 mL of buffer and filtered and concentrated a second time. Approximately 2 mL of the solution remained above the filter and was removed (LPCSF). Free sterol analyses were performed on the LPCS, LPCP, and on the protein concentrate, LPCSF.

ATPase Assay in the Presence or Absence of Surfactants

Ten to 20 μL of sample, containing 2.5 to 4 μg of PM protein, was used in a 500 μL assay mixture containing 5 mM $MgSO_4$, 50 mM KCl, 125 mM sucrose, 5 mM Na-ATP (pH 6.5), 0.1 mM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, and 30 mM Mes/Tris (pH 6.5) in the presence or absence of various concentrations of the surfactants Triton X-100, lysolecithin, sodium deoxycholate, and octylglucoside. The reaction proceeded at 37°C for 10 min and was stopped by the addition of 1 mL of stopping solution (1, 12). Pi was quantified by measuring the A_{660} of the solution 20 min after the addition of 50 μL of 10% (w/v) ascorbic acid.

Lipid Extraction

Lipids were extracted from plasma membrane preparations and surfactant treated membranes by the method of Bligh and Dyer (5). The total lipid extract was partitioned into neutral lipid, glycolipid, and phospholipid fractions on silica Sep-Pak cartridges (Millipore) (15, 25). Briefly, total lipid extracts were evaporated to near dryness under a stream of N_2, brought to a volume of 2 mL with chloroform:acetic acid (100:1 v/v), and transferred to the Sep-Pak cartridge attached to a 10 mL glass syringe. After the sample had entered the cartridge, 2 mL of chloroform:acetic acid (100:1 v/v) was used to wash residual lipid from the original container and transfer it to the cartridge. Ten mL of the same solvent were used to elute the neutral lipids from the column. Glycolipids were eluted by first adding 10 mL of acetone and subsequently 10 mL of acetone:acetic acid (100:1, v/v). Phospholipids were eluted using 10 mL of methanol:chloroform:water (100:50:40, v/v/v). Phospholipids were recovered from this fraction by the addition of 1 mL of chloroform and 2 mL of water to facilitate a phase separation.

The polar lipid fractions were chromatographed by HPTLC on precoated Silica Gel 60 plates (10 × 20 cm, Merck) using a mobile phase of chloroform:methanol:acetic acid:water (85:20:2:3, v/v/v/v). Fractionated lipids were identified by

co-chromatography with known lipids and detected by spraying with 6-*p*-toluidino-2-naphthalenesulfonic acid (18) and other specific spray reagents (20). Total lipid phosphorous as well as individual phospholipid phosphorous (scraped from the TLC plate) was analyzed by the method of Ames (1) after digestion in hot nitric acid. Total glycolipid was analyzed by the method of Roughan and Batt (32).

Total free sterol was determined by the colorimetric method of Zlatkis and Zak (39) using cholesterol as a standard. Individual sterols in the neutral lipid samples were quantified by GLC in a Varian 3700 gas chromatograph using 3% OV-17 on Gas Chrom Q (100/120 mesh) in a 6 ft by 2 mm (id) glass column under the following conditions: injection, column, and FID temperatures were 310, 270, and 350°C, respectively; N_2 carrier gas, air, and H_2 flow were 18 to 20, 300, and 30 ml/min, respectively. Signal output from the gas chromatograph was quantified using a Hewlett Packard 3390 A integrator. Retention times and response factors were determined by chromatography of known free sterol standards Summation of the individual sterol amounts from GLC gave values greater than those obtained from the colorimetric method described above.

Protein Determination

Protein was determined by the method of Lowry *et al.* (24) with modifications by Markwell *et al.* (26).

Electron Microscopy

Vesicle preparations, before and after Triton X-100 extraction, were incubated for 4 h in 4% glutaraldehyde in 25 mM Na-cacodylate buffer (pH 7.2), at room temperature. After rinsing 6 times over 2 h with buffer alone, the pellets were treated for 2 h in 2% OsO_4 in 25 mM cacodylate buffer (pH 7.2). The pellets were rinsed thoroughly with buffer, dehydrated in a graded ethanol series, placed in propylene oxide, and embedded in Epon. Silver/gold sections were cut and poststained for 15 min in saturated uranyl acetate while on copper grids. Electron micrographs were taken on a Jeol 100B electron microscope at 60 kV.

Assay Replication

Assays were repeated to obtain values for the mean ± SE. The number of replications are indicated where appropriate in the figure and table legends.

RESULTS

Four surfactants were tested for their ability to activate the U_3 ATPase. Of these four, three (Triton X-100, sodium deoxycholate, and octylglucoside) appeared to affect ATPase activity in a similar manner; they maximally activated the enzyme at specified optimal concentrations and inactivated it at superoptimal concentrations (Table I). The fourth surfactant, lysolecithin, elicited the highest specific activity, and differed from the other surfactants in that there was no inactivation of the enzyme at superoptimal levels. This is probably due to its inherent phospholipid character. The difference in effect on ATPase activity of lysolecithin *versus* Triton X-100 has been noted previously (33).

Table I. *Comparison of ATPase Specific Activities of Plasma Membrane Vesicles Treated with Four Different Surfactants*

The ATPase assay was initiated when approximately 4 μg of U_3 protein was added to 500 μL of the various buffered surfactant solutions. The values represent the means ± SE of two different assays.

Surfactant	Concentration	Concentration Status	ATPase Specific Activity	Percent of Initial
	% w/w		μmol/mg protein·min	
Triton X-100	0	Initial	0.9 ± 0.00	100
	0.0125	Optimal	3.1 ± 0.10	344
	0.060	Superoptimal	0.6 ± 0.03	67
Lysolecithin	0	Initial	0.6 ± 0.10	100
	0.01	Optimal	3.7 ± 0.30	617
	0.10	Superoptimal	3.2 ± 0.30	533
Sodium deoxycholate	0	Initial	0.8 ± 0.04	100
	0.03	Optimal	2.1 ± 0.10	263
	0.06	Superoptimal	0.6 ± 0.10	75
Octylglucoside	0	Initial	0.8 ± 0.10	100
	0.30	Optimal	1.7 ± 0.10	213
	0.5	Superoptimal	0.3 ± 0.02	38

Triton X-100 was used in this study to delipidate the PM. Purified PM vesicles were extracted with Triton X-100 at 25 to 1 (w/w) Triton to protein. This ratio was chosen because it gave maximal activation of the PM ATPase in the ATPase assay (33). Under the conditions used here, with a higher protein concentration than in the ATPase assay, this ratio is superoptimal for activation of the ATPase (data not shown). When the PM vesicles were treated with Triton X-100, and then centrifuged and washed, 41% of the protein was found to be insoluble (Table II). However, 100% of the total basal ATPase activity (assayed in the absence of surfactant) and 60% of the Triton-activated ATPase activity was still associated with the pellet. On the other hand, only 12% of the total lipid was still associated with the protein. The result was a decrease in μmol of lipid per mg protein from 0.8 to about 0.2. Despite the severe loss of lipids, the washed, Triton-insoluble material still possessed ATPase activity in the absence of added surfactants (Fig. 1) indicating that the lipid environment around the enzyme was still sufficient for activity. However, Triton X-100 still significantly increased the specific activity (Fig. 1), with maximum activation occurring at 0.005% (w/v), 2.5 times less than that needed to maximally activate the U_3 ATPase. The ATPase latency of the Triton-washed material was only 58% when compared to a 71% latency in the original U_3 PM fraction.

EM studies of the Triton-washed, pelleted material showed that some vesicles remained, despite the removal of most of the phospholipids. The original U_3 material consisted almost entirely of vesicles (Fig. 2, a and b), but in many cases the vesicles appeared to contain cytoplasmic contaminants. In contrast, the material after Triton extraction contained more membrane sheets and disrupted vesicles (Fig. 2, c and d); however, the vesicles that remained appeared to be free of

Table II. *Comparison of Protein, ATPase Activity, and Total Lipids in Oat Root Plasma Membranes before (U_3) and after (TXP) Extraction with Triton X-100 at a Ratio of 25 to 1, w/w, Triton to U_3 Protein*

Total lipid amounts represent the summation of total phospholipids, glycolipids, and free sterols. Values for protein, ATPase activity, and lipids represent the mean ± SE from 3 to 14 different preparations. ATPase was assayed in the absence of surfactant, presence of optimal Triton X-100, 0.0125%, w/v, for U_3 and 0.005%, w/v, for TXP.

Assay	U_3	TXP	Percent Change
Protein (mg)	2.0 ± 0.0	0.83 ± 0.05	−59
Specific activity (μmol Pi/mg protein·mg)			
Basal	0.9 ± 0.1	2.0 ± 0.3	+122
+ Triton	3.2 ± 0.2	4.8 ± 0.4	+50
Total activity (μmol Pi/min)			
Basal	1.7 ± 0.1	1.7 ± 0.2	0
+ Triton	6.3 ± 0.5	4.0 ± 0.6	−37
Total lipid			
μmol	1.7 ± 0.04	0.2 ± 0.01	−88
μmol/mg protein	0.8 ± 0.02	0.2 ± 0.02	−75

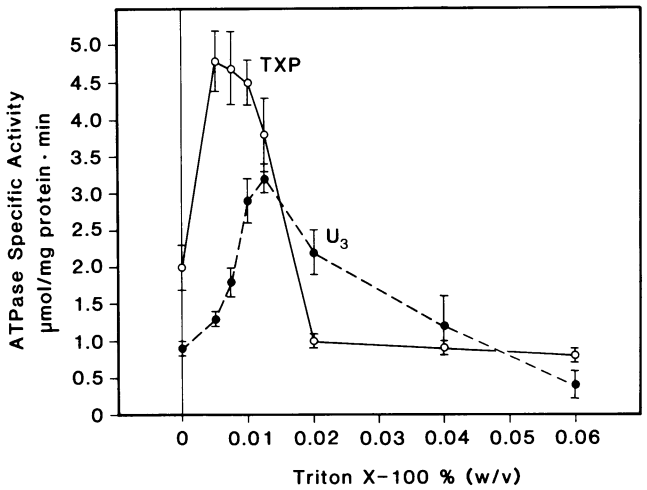

Figure 1. Effect of different Triton X-100 concentrations on PM ATPase specific activity from PM treated with Triton X-100 (25 to 1, Triton to PM protein, *open circles*) and untreated PM (*closed circles*). Samples of 0.5 mL containing 4 μg of PM protein were assayed as described in "Materials and Methods." Values represent the mean of triplicate samples.

cytoplasmic contaminants. A significant portion of the protein that is lost from the U_3 vesicles (60%), upon treatment with Triton, may be from the cytoplasmic contaminants that have been released from the vesicle interiors rather than true PM proteins. Significant numbers of multilaminar vesicles were not observed, in contrast to the situation with sarcoplasmic reticulum vesicles after a Triton X-100 treatment (29).

An analysis was then carried out on the lipids remaining associated with the insoluble ATPase preparation after Triton X-100 extraction, and the data are shown in Table III. Only a summation of the three main classes of lipids is shown here; a complete characterization of each of these lipid classes in

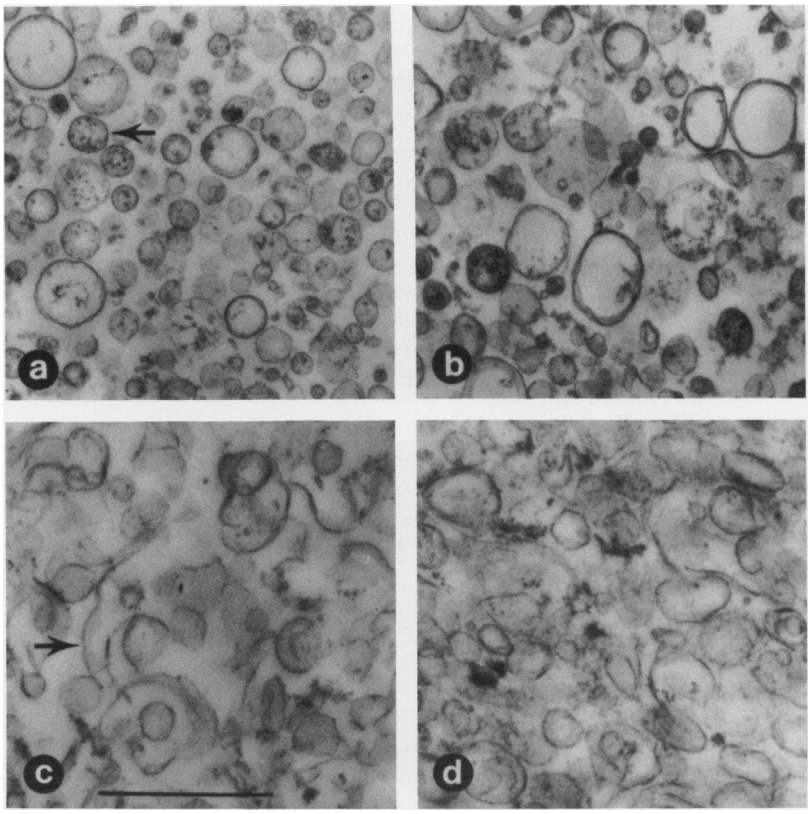

Figure 2. Appearance of PM vesicles preparations before and after Triton X-100 extraction as viewed in the electron microscope. Membranes fixed in glutaraldehyde, stained with OsO_4, and poststained with uranyl acetate. (a and b) Untreated U_3 vesicle preparation. Vesicles appear intact and unilaminar. Note that many vesicles appear to contain cytoplasmic contaminants (*arrow* in a). (c and d) Vesicles after extraction with 25 to 1 (w/w) Triton to protein and subsequent high speed centrifugation. Note that while some intact vesicles are apparent, in many cases the material appears as sheets (*arrow* in c). Bar: 1 μm.

Table III. *Comparison of Lipid Components in Oat Root Plasma Membranes before (U_3) and after (TXP) Extraction Triton X-100 at a Ratio of 25 to 1, w/w, Triton to U_3 Protein*

Lipids were extracted, separated into total phospholipids, glycolipids, and free sterols, and quantified using standard colorometric techniques as described in "Materials and Methods." Protein before extraction was 2.0 mg, after extraction 0.83 mg. Values represent the means ± SE from 7 to 14 different PM preparations.

Lipid Component	U_3	TXP	Percent Change	U_3	TXP	Percent Change
	μmol/mg protein			*mol % of total lipid*		
Phospholipid	0.38 ± 0.03	0.06 ± 0.01	−84	45	25	−44
Glycolipid	0.26 ± 0.02	0.10 ± 0.02	−62	31	42	+35
Free sterol	0.21 ± 0.01	0.08 ± 0.01	−62	25	33	+32

oat root PM vesicles is presented elsewhere (32a). The most striking effect of Triton extraction was the reduction in the phospholipids (lipid phosphorous), whose concentration (μmol/mg protein) was reduced by 84%. Glycolipids (lipid sugar) and sterols were each reduced by 62%. If one assumes that the average mol wt of the PM proteins is 50,000, a ratio of each of these lipid molecules to protein can be calculated. Before extraction (*i.e.* in the U_3), there were approximately 19 phospholipid, 13 glycolipid, and 11 sterol molecules associated, on average, with each protein molecule. In fact, the ratios must be even higher, since some of the protein may be cytoplasmic contaminants. On the other hand, only 3 phospholipid, 5 glycolipid, and 4 sterol molecules were associated with each molecule of protein after extraction.

While Triton X-100 differentially extracted the different classes of lipids, it also caused some differential extraction of components within a lipid class. In the intact PM vesicles the principal phospholipids were phosphatidylcholine and phosphatidylethanolamine with smaller amounts of phosphatidic acid, phosphatidylserine, and phosphatidylinositol (32a). When the small amount of phospholipids remaining after Triton X-100 treatment were separated by HPTLC, only phosphatidylcholine and phosphatidylethanolamine could be detected. HPTLC of the glycolipid fraction from Triton-treated PM vesicles showed that the detectable components were predominantly the ones containing sterol (steryl glycosides and acylated steryl glycosides). Glycocerebrosides, which exist in intact oat root PM (32a), could not be detected in the Triton-insoluble material. In intact oat root PM fractions, the

Table IV. *Free Sterols Associated with Protein Fractions after Partial Delipidation by Surfactants (see "Materials and Methods" for Details)*

U_3 represents the untreated plasma membrane. TXP represents the pellet remaining after treatment of U_3 with Triton X-100 at a ratio of 25 to 1 (Triton to U_3 protein). LPCP represents the pellet remaining after treatment of TXP with 0.1%, w/v, lysolecithin. LPCSF represents the protein concentrate remaining after filtering the supernatant from the lysolecithin treated TXP through a membrane with a mol wt exclusion limit of 100,000. Values represent the mean ± SE of two to four separate PM preparations.

Free Sterol	U_3	TXP	LPCP	LPCSF
	mol % of total sterol			
Cholesterol	0.5 ± 0.1	2.7 ± 0.8	1.0 ± 0.1	3.3 ± 0.4
Campesterol	8.2 ± 0.4	10.5 ± 1.1	8.6 ± 0.1	13.9 ± 0.5
Stigmasterol	48.2 ± 2.8	45.4 ± 1.6	52.1 ± 1.0	44.1 ± 0.8
β-Sitosterol	21.3 ± 0.8	23.4 ± 1.4	23.1 ± 0.5	25.8 ± 0.4
Unknown[a]	21.8 ± 2.3	18.1 ± 1.6	15.2 ± 1.5	13.1 ± 1.2

[a] Tentatively identified as a cyclopropyl sterol.

principal sterols (Table IV) were stigmasterol, β-sitosterol, and an unknown, tentatively identified as a cyclopropyl sterol related to cycloartenol (32a). Campesterol was present in smaller amounts, with only a trace of cholesterol. The Triton X-100 extraction removed the sterols proportional to their mol %. Only cholesterol showed a significant enrichment with Triton treatment. The extraction by Triton X-100, then, left protein (enriched in PM-ATPase) with reduced levels of lipids. However, the proportion of sterols (free sterols + glycolipids) in the lipid phase was significantly increased, along with an increase in ATPase specific activity, which was probably due to loss of other protein constituents.

Lysolecithin (LPC) is known to solubilize the PM-ATPase (31). To determine whether the sterols are also solubilized by LPC and remain associated with the proteins, the Triton-insoluble pellet was extracted with 0.1%, w/v, LPC. This solubilized about 50% of the protein, including most of the ATPase, and about 25% of the sterols (Table V). Only sterols were analyzed at this point because the glycolipids and phospholipids are not easily or satisfactorily quantified in such micro quantities. The solubilized protein was subjected to filtration through a membrane exclusion filter, which we determined allows about 90% of an unbound sterol-lysolecithin mixture to pass through (data not shown). All of the

Table V. *Comparison of Protein, ATPase Activity, and Individual Free Sterols in Oat Root Plasma Membranes before and after Extraction with Triton X-100 (25 to 1, w/w, Triton to U_3 Protein) and Subsequently Lysolecithin (25 to 1, w/w, Lysolecithin to TXP Protein)*

Analyses (see "Materials and Methods") were performed on untreated PM (U_3), the pellet from Triton X-100 treated U_3 (TXP), the supernatant (LPCS) and pellet (LPCP) from TXP, and the same lysolecithin supernatant that was filtered (LPCSF) through an Amicon XM-100 filter (mol wt exclusion limit of 100,000). Values represent the mean ± SE of two to four separate PM preparations.

	U_3	TXP	LPCP	LPCS	LPCSF
Protein (mg)	4.0 ± 0.0	1.8 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.3 ± 0.0
ATPase activity					
Specific (μmol/mg protein·min)	3.8 ± 0.5	4.8 ± 0.4	0.9 ± 0.3	6.3 ± 0.5	2.1 ± 1.9
Total (% of U_3)	100	49	4	31	4
Total sterol[a] (μmol)	1.11 ± 0.05	0.13 ± 0.02	0.12 ± 0.01	0.039 ± 0.028	0.052 ± 0.010

[a] Addition of individual sterols quantified by GLC.

sterols remained in the concentrate, along with 40% of the protein. ATPase, with a mol wt of about 100,000 (34), would be expected to remain in the concentrate, and was, indeed, found there, although it appeared to have been substantially inactivated. In general, the spectrum of sterols which remain associated with the ATPase-protein fraction after LPC solubilization is similar to that in the original U_3 membranes (Table IV), although there has been a concentration of cholesterol and to a lesser extent campesterol, and a loss of the unknown sterol.

DISCUSSION

The plasma membrane is a complex mixture of proteins and a variety of lipid components. Treatment of PM vesicles with surfactants results in the removal of considerable lipid material and the solubilization of some of the protein. In addition, the activity of the U_3 PM-ATPase is enhanced up to five-fold by surfactants (33). In this study we have addressed the question concerning the amounts and types of lipids that remained associated with the ATPase after surfactant treatment, and the possible effects of lipid components on ATPase activity. The amount of Triton X-100 used here (the ratio of Triton to protein used in the ATPase assay that gives maximum activity) solubilized nearly 60% of the protein. This would be an overestimate of the amount of PM protein that is solubilized, if, as it appears from the EM pictures, the Triton wash has removed considerable trapped, protein-containing cytoplasmic components. In addition, the Triton treatment removed almost 90% of the lipid. Only a small amount of phospholipid remained associated with the ATPase-containing pellet, but a larger amount of glycolipid (mostly sterol derivatives) and free sterol remained. This suggests that there is no absolute requirement for phospholipids for ATPase activity. This might seem to contradict the frequent observations that after surfactant treatment, ATPase activity can be at least partially restored by addition of phospholipids (2, 8, 9, 17, 19). Studies with other membrane-bound ATPases have shown, however, that as long as an adequate hydrophobic environment with sufficient fluidity is maintained around the ATPase, by either surfactants or lipids, activity is maintained or enhanced depending on anomalies in that environment (4, 11, 16, 23, 27). Palmgren *et al.* (28) have suggested that the enhancement of oat root PM ATPase by LPC and free fatty acids is due to a change in membrane fluidity. However, it is reasonable to believe that any changes in membrane fluidity could be reflected in the properties of the lipids immediately surrounding the ATPase. Triton X-100 or lysolecithin may simply be superior to the native phospholipids in maintaining the required membrane fluidity or protein configuration for ATPase activity.

The amount of sterols and sterol-derivatives remaining with the ATPase fraction was surprising. When the insoluble ATPase was solubilized with LPC, 30% of the sterols remained associated with that fraction and were not removed with filtering. In fact, the association between the sterols and the ATPase may be difficult to break. We tested the more severe delipidation procedure of Anton and Spanswick (2) to see if this would result in ATPase free of sterols. The U_3 vesicles were extracted with Triton X-100 (0.1%, w/w) followed by octylglucoside (1%, w/w) and sodium deoxycholate (0.2%, w/w). The resulting pellet was suspended in lysolecithin (1.0%, w/w) to solubilize the ATPase. After high speed centrifugation to remove insoluble material, we extracted the free sterols from the lysolecithin supernatant (the same material that would have been loaded onto the glycerol gradient in their study). A significant amount of the total free sterol is recovered in this fraction (data not shown) again suggesting that complete surfactant mediated delipidation may not be possible and sterols are associated with active PM ATPase.

What is the role of the sterols in ATPase activity? Studies with other ATPases have suggested that sterols alter ATPase activity by influencing the fluidity of the membrane (6, 7, 10, 30, 37, 38). However, the free sterols may also function to preserve the configuration of the enzyme and help modulate the interaction between the enzyme and phospholipids or surfactants. In a practical sense, this may serve to protect the ATPase from permanent denaturation during extraction and facilitate or enhance its activity during reconstitution. Changes in the sterols associated with the ATPase may also help determine the maximum activity of the enzyme. Thus the different maximum PM-ATPase activities reported for oat roots and oat coleoptiles (32a) or between U_3 preparations of oat roots and other plant tissues from different laboratories (Fig. 1) (3, 22, 36) may be due, in part, to the sterols associated with the ATPase.

It has frequently been suggested that the activation of the PM-ATPase by low levels of surfactants is simply due to a breakdown in vesicle impermeability to ATP, with the result that ATP now reaches the active site of the ATPase in right-side out vesicles. This idea has been strongly supported (3, 13, 14, 22). We have suggested from ESR data (33), on the other hand, that the surfactants may directly activate the ATPase in addition to altering vesicle permeability.

Palmgren *et al.* (28) have now reached the same conclusion as a result of their studies on fatty acid and lysolecithin activation of PM ATPase from oat roots.

We have shown that even though the 25 to 1 (w/w) Triton to protein extraction removed 85% of the phospholipids, the remaining insoluble ATPase was still increased in activity by low levels of Triton X-100 and showed an apparent latency of 57%. Could this latency be explained as simply due to an increased permeability of sealed vesicles to ATP? This seems unlikely. Examination of the Triton-washed pellet with the EM showed that some vesicles still remained (Fig. 2b). However, the proportion of vesicular material was considerably reduced, while the apparent latency was only reduced from 71% to 57%. The fact that any vesicular material can be seen is of interest, in light of the great reduction in phospholipid to protein ratio from greater than 19 to 1 to only 3 to 1 after Triton treatment. What, then, makes up the vesicle membranes that are apparent in the electronmicrographs? One possibility is that Triton X-100 has remained associated with the membrane and is not removed by washing as suggested by Galtier *et al.* (13).

The Triton X-100 to protein ratio present during the treatment of vesicles with surfactants apparently has a large effect on how much vesicle lipid and protein is removed. Galtier *et al.* (13) have shown that treatment of corn root PM vesicles

with Triton to protein ratios between 4 to 1 and 1 to 1 resulted in no decrease in the number of tightly sealed vesicles, but apparently caused a reorientation of some of the ATPase molecules so that half were right-side out and the other half were inside out. This resulted in a great increase in ATP-driven proton transport capacity after Triton treatment. A similar increase in proton transport capacity has been seen after treatment of pea root vesicles with deoxycholate (RE Cleland, unpublished data). The treatment of our oat root PM vesicles with the higher Triton to protein ratio resulted in material that had no measurable ATP-driven proton transport (data not shown), consistent with the idea that tightly sealed vesicles no longer existed. It should be noted that Prado *et al.* (29) showed that while 10^{-5} M Triton X-100 rendered sarcoplasmic reticulum vesicles permeable to EM negative stain, it did not alter the Ca^{2+}-ATPase activity. At 10^{-4} M, on the other hand, membrane ATPase activity was greatly increased without obvious alteration of the vesicles as viewed in the EM. Our data support the idea, then, that surfactants can directly activate PM ATPases, in addition to altering vesicle permeability to ATP.

ACKNOWLEDGMENTS

The authors thank Douglas Tayler for preparing the electron micrographs, Paul Linnemeyer for the ATP-driven proton transport work and Drs. A. deBoer and D. Lynch for helpful discussions.

LITERATURE CITED

1. **Ames BN** (1966) Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol **8:** 115–118
2. **Anton GE, Spanswick RM** (1986) Purification and properties of the H^+-translocating ATPase from the plasma membrane of tomato roots. Plant Physiol **81:** 1080–1085
3. **Berczi A, Moller IM** (1987) Mg^{2+}-ATPase activity in wheat root plasma membrane vesicles: time-dependence and effect of sucrose and detergents. Physiol Plant **70:** 583–589
4. **Bigelow DJ, Thomas DD** (1987) Rotational dynamics of lipid and the Ca-ATPase in sarcoplasmic reticulum: the molecular basis of activation by diethyl ether. J Biol Chem **262:** 13449–13456
5. **Bligh EG, Dyer WJ** (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol **37:** 911–917
6. **Bloch K** (1984) Cholesterol: Evolution of structure and function. *In* DE Vance, JE Vance, eds, Biochemistry of Lipids and Membranes. Benjamin/Cummings, Menlo Park, CA, pp 1–24
7. **Borchman D, Simon R, Bicknell-Brown E** (1982) Variation in the lipid composition of rabbit muscle sarcoplasmic reticulum membrane with muscle type. J Biol Chem **257:** 103–109
8. **Cocucci M, Ballarin-Denti A** (1981) Effect of polar lipids on ATPase activity of membrane preparations from germinating radish seeds. Plant Physiol **68:** 377–381
9. **Cocucci MC, Marre E** (1984) Lysophosphatidylcholine-activated, vanadate-inhibited, Mg^{2+}-ATPase from radish microsomes. Biochim Biophys Acta **771:** 42–52
10. **Douglas TJ, Walker RR** (1984) Phospholipids, free sterols and adenosine triphosphatase of plasma membrane-enriched preparations from roots of citrus genotypes differing in chloride exclusion ability. Physiol Plant **62:** 51–58
11. **Dufour JP, Goffeau A** (1980) Phospholipid reactivation of purified plasma membrane ATPase of yeast. J Biol Chem **255:** 10591–10598
12. **Dulley JR** (1975) Determination of inorganic phosphate in the presence of detergents or protein. Anal Biochem **67:** 91–96
13. **Galtier N, Belver A, Gibrat R, Grouzis J-P, Rigaud J, Grignon C** (1988) Preparation of corn root plasmalemma with low Mg-ATPase latency and high electrogenic H^+-pumping activity after phase partitioning. Plant Physiol **87:** 491–497
14. **Grouzis J-P, Gibrat R, Rigaud J, Grignon C** (1987) Study of sidedness and tightness to H^+ of corn root plasmalemma vesicles: preparation of a fraction enriched in inside-out vesicles. Biochim Biophys Acta **903:** 449–464
15. **Hamilton JG, Comai K** (1984) Separation of neutral lipids and free fatty acids by high performance liquid chromatography using low wave-length ultraviolet detection. J Lipid Res **25:** 1142–1148
16. **Hidalgo C, Thomas DD, Ikemoto N** (1978) Effect of the lipid environment on protein motion and enzymatic activity of the sarcoplasmic reticulum calcium ATPase. J Biol Chem **253:** 6879–6887
17. **Imbrie CW, Murphy TM** (1984) Photoinactivation of detergent-solubilized plasma membrane ATPase from *Rosa damascena*. Plant Physiol **74:** 617–621
18. **Jones M, Keenan RW, Horowitz P** (1982) Use of 6-*p*-toluidino-2-naphthalenesulfonic acid to quantitate lipids after thin-layer chromatography. Chromatography **237:** 522–524
19. **Kasamo K, Nouchi I** (1987) The role of phospholipids in plasma membrane ATPase in *Vigna radiata* L. (mung bean) roots and hypocotyls. Plant Physiol **83:** 323–328
20. **Kates M** (1972) Techniques of lipidology. *In* TS Work, E Work, eds, Laboratory Techniques in Biochemistry and Molecular Biology, Vol 3, Part 2. Elsevier/North-Holland, Amsterdam, pp 265–610
21. **Keenan TW, Leonard RT, Hodges TK** (1973) Lipid composition of plasma membranes from Avena sativa roots. Cytobios **7:** 103–112
22. **Larsson C** (1985) Plasma membranes. *In* HF Linskens, JF Jackson, eds, Modern Methods of Plant Analysis (New Series), Vol 1: Cell Components. Springer-Verlag, Berlin, pp 85–104
23. **Le Marie M, Moller JV, Tanford C** (1976) Retention of enzyme activity by detergent-solubilized sarcoplasmic Ca^{2+}-ATPase. Biochemistry **15:** 2336–2342
24. **Lowry OH, Rosebrough NJ, Farr AL, Randal RJ** (1951) Protein measurement with the folin phenol reagent. J Biol Chem **193:** 265–275
25. **Lynch DV, Steponkus PL** (1987) Plasma membrane lipid alterations associated with cold acclimation of winter rye seedlings (*Secale cereale* L. cv Puma). Plant Physiol **83:** 761–767
26. **Markwell MAK, Haas SM, Bieber LL, Tolbert NE** (1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem **87:** 206–210
27. **McIntosh DB, Ross DC** (1985) Role of phospholipid and protein-protein associations in activation and stabilization of soluble Ca^{2+}-ATPase of sarcoplasmic reticulum. Biochemistry **24:** 1244–1251
28. **Palmgren MG, Sommarin M, Ulvskov P, Jorgensen PL** (1988) Modulation of plasma membrane H^+-ATPase from oat roots by lysophosphatidylcholine, free fatty acids and phospholipase A_2. Physiol Plant **74:** 11–19
29. **Prado A, Arrondo JLR, Villena A, Goni FM, Macarulla JM** (1983) Membrane surfactant interactions: the effect of Triton X-100 on sarcoplasmic reticulum vesicles. Biochim Biophys Acta **733:** 163–171
30. **Proverbio F, Proverbio T, Rawlins F** (1980) Effect of partial substitution of membrane cholesterol by other sterols on the sodium-potassium ATPase activity of microsomal fractions from rat brain cortex. Acta Cient Venez **31:** 219–223
31. **Rochester CP, Kjellbom P, Andersson B, Larsson C** (1987) Lipid composition of plasma-membranes isolated from light-grown barley (*Hordeum vulgare*) leaves: identification of cerebroside as a major component. Arch Biochem Biophys **255:** 385–391
32. **Roughan PG, Batt RD** (1968) Quantitative analysis of sulfolipid (sulfoquinovosyl diglycerides) and galactolipids (monogalactosyl and digalactosyl diglycerides) in plant tissues. Anal Biochem **22:** 74–88

32a. **Sandstrom RP, Cleland RE** (1989) Comparison of the lipid composition of oat root and colcoptile plasma membranes.

Lack of short-term change in response to auxin. Plant Physiol **90:** 1207–1213

33. **Sandstrom RP, DeBoer AH, Lomax TL, Cleland RE** (1987) Latency of plasma membrane H^+-ATPase in vesicles isolated by aqueous phase partitioning: increased substrate accessibility or enzyme activation? Plant Physiol **85:** 693–698
34. **Serrano R** (1984) Purification of the proton pumping ATPase from plant plasma membranes. Biochem Biophys Res Commun **121:** 735–740
35. **Serrano R** (1985) Plasma Membrane ATPase of Plants and Fungi. CRC Press, Boca Raton, FL, pp 1–174
36. **Sommarin M, Lundborg T, Kylin A** (1985) Comparison of K,Mg ATPases in purified plasmalemma from wheat and oat. Substrate specificities and effects of pH, temperature and inhibitors. Physiol Plant **65:** 27–32
37. **Xie XS, Tsai SJ, Stone DK** (1986) Lipid requirements for reconstitution of the proton-translocating complex of clathrin-coated vesicles. Proc Natl Acad Sci USA **83:** 8913–8917
38. **Yoda S, Yoda A** (1987) Phosphorylated intermediates of Na, K-ATPase proteoliposomes controlled by bilayer cholesterol: Interaction with cardiac steroid. J Biol Chem **262:** 103–109
39. **Zlatkis A, Zak B** (1969) Study of a new cholesterol reagent. Anal Biochem **29:** 143–148