Plant Physiol. (1987) 85, 693-698
0032-0889/87/85/0693/06/$01.00/0

Latency of Plasma Membrane H^+-ATPase in Vesicles Isolated by Aqueous Phase Partitioning[1]

INCREASED SUBSTRATE ACCESSIBILITY OR ENZYME ACTIVATION

Received for publication April 6, 1987 and in revised form July 17, 1987

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ABSTRACT

The properties of the plasma membrane H^+-ATPase and the cause of its latency have been studied using a highly purified plasma membrane fraction from oat (*Avena sativa* L., cv Victory) roots, prepared by aqueous two-phase partitioning. The ATPase has a maximum specific activity (at 37°C) in excess of 4 micromoles inorganic phosphate per milligram protein per minute in the presence of nondenaturing surfactants. It is inhibited by more than 90% by vanadate, is specific for ATP, has a pH optimum of 6.5, and is stimulated more than 4-fold by 50 millimolar K^+ in the presence of low levels of the nondenaturing surfactants Triton X-100 and lysolecithin. This 'latent' activity is usually explained as being a result of the inability of ATP to reach the ATPase in right-side out, sealed vesicles, until they are disrupted by surfactants. Consistent with this idea, trypsin digestion significantly inhibited the ATPase only in the presence of the surfactants. Electron spin resonance spectroscopy volume measurements confirmed that surfactant-free vesicles were mostly sealed to molecules similar to ATP. However, the Triton to protein ratio required to disrupt vesicle integrity completely is 10-fold less than that needed to promote maximum ATPase activity. We propose that plasma membrane ATPase activation is due not solely to vesicle disruption and accessibility of ATP to the ATPase but to the surfactants activating the ATPase by altering the lipid environment in its vicinity or by removing an inhibitory subunit.

The plant PM[2] H^+-ATPase is involved in a number of crucial plant functions including acidification of cell walls (which facilitates cell elongation), regulation of cytoplasmic pH, and maintenance of the protonmotive force (which is necessary for the membrane transport of ions and sugars) (24, 26, 28). *In vivo*, plant cell wall acidification is stimulated by the plant growth hormone auxin and by the fungal toxin fusicoccin, suggesting that the PM H^+-ATPase is under some type of hormonal regulation (20), but the mechanisms remain obscure.

ATPase action has frequently been studied using vesicles prepared by sucrose or dextran density-gradient centrifugation (5, 7, 27, 28). This method yields sealed, inside-out PM vesicles in which access of ATP to the ATPase is not a problem, and in which ATP-driven proton transport can be studied. However, the preparation also contains significant amounts of extraneous, contaminating membrane vesicles, and the maximum ATPase activity is generally low (<1 μmol $Pi \cdot mg^{-1}$ protein $\cdot min^{-1}$). A much purer PM vesicle fraction can be obtained by aqueous two-phase partitioning (7, 9, 10, 25, 30). With such fractions, the ATPase activity is usually significantly enhanced by surfactants. This has been interpreted (9, 10) as indicating that the vesicles are sealed and right-side out and that the latency is due to the increased accessibility of ATP to the ATPase when the vesicles are opened by the surfactant.

In this study, the aqueous two-phase system has been used to obtain a highly purified PM vesicle fraction from oat roots. This fraction, which has an unusually high ATPase activity, has been used to examine the basis of latency for the PM ATPase.

MATERIALS AND METHODS

Chemicals. Sodium-ATP, IDP, dextran (mol wt 500,000), polyethylene glycol (mol wt 4,000), porcine trypsin (T0134), and trypsin inhibitor were purchased from Sigma. ADP, GDP, UDP, CDP, GTP, UTP, and CTP were purchased from Boehringer Mannheim Biochemicals. All other chemicals were analytical reagent grade.

Plant Material. Oat (*Avena sativa* L. cv Victory) seeds were rinsed in running tap water for approximately 20 min, sown on cheese cloth covering a stainless steel screen 1 cm over a 1 mM $CaSO_4$ solution, and grown for 5 d in continuous (dim) red light at 25°C. The solution was changed daily for the final 3 d. At harvest, the roots were usually from 8 to 10 cm in length.

Extraction of Membrane Vesicles. A razor blade was used to harvest those roots protruding from below the screen. All subsequent steps were at 0 to 4°C. The roots (200 g) were ground in a Waring blender containing 400 ml of buffer I: 10 mM Tris/HCl (pH 7.5), 250 mM sucrose, and 1 mM EDTA. Protease inhibitors were found to be unnecessary (data not shown) and therefore not used. The homogenate was filtered through 4 layers of cheesecloth and centrifuged for 20 min at 10,000*g* in a fixed angle rotor (r_{max} = 10.5 cm). The resulting supernatant was centrifuged for 30 min at 100,000*g* to obtain the microsomal pellet.

Aqueous Phase Partitioning. The aqueous phase partitioning procedure has recently been reviewed by Larsson (10). Briefly, the microsomal pellet from 200 g of roots was resuspended in buffer II (5 mM potassium phosphate [pH 7.8], 250 mM sucrose, and 4 mM KCl) and brought to a final weight of 5 g. Three tubes comprising the aqueous phase partition system were made as follows: tube A contained 15 g of a phase system that would contain, after addition of the 5 g sample, 6.5% (w/w) dextran (500,000 mol wt), 6.5% (w/w) polyethylene glycol (4,000 mol

[1] Supported by the National Science Foundation, Grant DMB-8502021, and the Department of Energy, Contract DE-AT06-76ER73019

[2] Abbreviations: PM, plasma membrane; ESR, electron spin resonance; U_3, upper phase from tube 3 of an aqueous phase partitioning system; T:p, Triton X-100 to protein ratio; PCAol, 2,2,5,5-tetramethyl-3-methanolpyrroline-1-oxyl; TEPA, Ni-tetraethylenepentamine

wt), 250 mM sucrose, 5 mM potassium phosphate (pH 7.8), and 4 mM KCl. Tubes B and C contained 10 g of the lower phase obtained by partitioning a complete phase system identical in composition and final concentration to tube A. After the microsomal suspension was layered onto the phase system in tube A, it was mixed by inversion 30 to 40 times and centrifuged in a swinging bucket rotor at 1,000*g* for 5 min. Ninety to 95% of the resulting upper phase (U_1) was transferred to tube B, and the mixing and centrifugation were repeated. The procedure was repeated again for tube C. To remove the polyethylene glycol, the resulting upper layer (U_3) was added to approximately 10 ml of buffer III (10 mM Tris/HCl (pH 7.5), and 250 mM sucrose) and centrifuged for 30 min at 100,000*g*. The resulting pellet was resuspended in buffer III to give a final protein concentration of 0.3 to 7.0 mg/ml and stored at −70°C.

ATPase Assay. The resuspended plasma membrane fraction (U_3; 10 μl containing 2–2.5 μg of protein) was added to 490 μl of assay buffer containing 5 mM $MgSO_4$, 50 mM KCl, 125 mM sucrose, 5 mM NaATP (pH 6.5), 0.1 mM $(NH_4)_6Mo_7O_{24}\cdot 4\ H_2O$, and 30 mM Mes/Tris (pH 6.5) in the presence and absence of 0.0125% (w/v) Triton X-100; the Triton to protein ratio (T:p) was approximately 25. The reaction proceeded at 37°C for 20 min and was stopped by adding 1 ml of a solution containing 2% concentrated H_2SO_4, 5% SDS, and 0.5% $(NH_4)_6Mo_7O_{24}\cdot 4\ H_2O$). Total Pi from ATP hydrolysis was determined spectrophotometrically by adding 50 μl of 10% ascorbic acid and determining the A_{660} after 20 min (1, 4). Activity was expressed in terms of μmol Pi consumed per mg protein per min as the average of the time measured. Additions and modifications to the ATPase reaction mixture included various concentrations of Triton X-100, lysolecithin, salts, nucleotides, and buffers, as given in the figure legends. Triplicate samples were run for each assay. The rate of ATP hydrolysis was found to drop off slightly after 20 min; therefore, subsequent assays were incubated for 10 min and are noted where appropriate.

Enzyme Assays. Cyt *c* oxidase was estimated according to the procedure of Tolbert (29). Antimycin A-resistant NADH Cyt *c* reductase was measured according to the procedure described by Lord (12). Latent IDPase was estimated by the method described by Nagahashi and Nagahashi (17). Nitrate-sensitive ATPase was assayed according to the procedure of Mandala and Taiz (15).

Sucrose Density Gradient Centrifugation. One-half ml of U_3 containing approximately 0.75 mg of total protein was layered onto a linear 20 to 55% sucrose gradient and centrifugated at 140,000*g* in a swinging bucket rotor (r_{max} = 16.6 cm) at 4°C for 4 h. Fractions of 0.5 ml were collected and monitored for protein (by absorbance at 280 nm), ATPase activity, and sucrose concentration (by refractometry).

Trypsin Experiments. PM vesicle samples containing about 0.2 μg/μl of total protein were treated with or without trypsin (1:2 w/w, trypsin:PM protein) for 5 min at 25°C in 100 mM Tris/HCl (pH 8.0) and 125 mM sucrose, in the presence or absence of Triton X-100 (0.0125% w/v). Trypsin inhibitor was used at a ratio of 1.5:1 w/w (inhibitor:trypsin).

ESR Spectroscopy. The absolute volume of the inner aqueous phase of the oat root PM vesicles was evaluated by ESR spectroscopy using the nitroxide spin probe, PCAol (1 mM) with a Varian E-109 E spectrometer at a microwave power of 10 mW and modulation amplitude of 0.4 G. Vesicles at a concentration of 2.12 mg protein·ml^{-1} were mixed with increasing concentrations of Triton X-100 and 3 μl of a 1.75 M quencher solution; Ni-TEPA (Ni-tetraethylenepentamine, pH 7.8). The sample, contained in a 75 μl capillary tube, had a final volume of 45 μl. The quenched aqueous signal was expressed as a fraction of the unquenched signal and converted to the sealed volume contained in the total assay volume as explained in detail by Lomax and Mehlhorn (13).

Protein Determination. Protein was determined by the method of Lowry *et al.* (14) with modifications by Markwell *et al.* (16) and Peterson (18).

RESULTS

The U_3 fraction from aqueous phase partitioning of oat root membranes was characterized as to PM purity by determining the amount of marker enzymes for other contaminating membranes (Table I). The data indicate that contamination by membranes other than PM was minimal and correspond well with other studies where aqueous phase partitioning was used to obtain PM vesicles of high purity (7, 9, 10, 23, 30). For example, the nitrate-inhibited ATPase which has been localized to the tonoplast (15), was only about 1% of the vanadate-sensitive ATPase activity. Latent IDPase, considered to be a Golgi-associated enzyme (17), was also found in small amounts in the U_3 fraction. The absence of Golgi and tonoplast was further suggested by the near lack of a pyrophosphatase in the U_3 fraction (Table II). In striking contrast, however, was the unusually high activity of the Mg^{2+}-, K^+-stimulated, vanadate inhibited ATPase, which was obtained in the presence of 0.0125% (w/v, T:p = 25) Triton X-100. The U_3 ATPase specific activity (shown in Table I, 4.0 μmol·mg^{-1}·min^{-1}) is 8 times greater than that found in microsomal preparations (0.5 μmol·mg^{-1}·min^{-1}) under the same assay conditions. This indicates that phase partitioning has resulted in a large enrichment and purification for vesicles of

Table I. *Specific Activities of Selected Membrane Markers*

Assays were conducted on microsomal and U_3 PM vesicles isolated by phase partitioning from 5 d old oat roots. The K^+, Mg^{2+}-ATPase assays contained 5 mM $MgSO_4$, 50 mM KCl, 125 mM Sucrose, 5 mM NaATP (pH 6.5), 0.1 mM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 30 mM Mes/Tris (pH 6.5), and 0.0125% (W/V) Triton X-100. Assay temperature was 37°C. The NO_3-sensitive ATPase contained the same components as the K^+, Mg^{2+}-ATPase plus 50 mM $NaNO_3$ and assayed at pH 7.0. Values represent the means ± SE; n = 4.

Enzyme	Fraction	Specific Activity	
		Microsomal	U_3
		μmol·mg^{-1} protein·min^{-1}	
NADH Cyt *c* reductase	ER	0.14 ± 0.02	0.02 ± 0.00
Cyt *c* oxidase	Mitochondria	0.67 ± 0.04	0.00 ± 0.00
K^+, Mg^{2+}-ATPase (vanadate sensitive) + 0.0125% (w/v) Triton X-100	PM	0.49 ± 0.13	4.00 ± 0.02
Latent IDPase	Golgi	0.08 ± 0.02	0.02 ± 0.00
NO_3^--sensitive ATPase	Tonoplast	0.05 ± 0.02	0.06 ± 0.03

Table II. *Substrate Specificity of the U_3PM ATPase*

Assays were run, as described in Figure 1, with 5 mM of each nucleotide. Values represent the means ± SE; n = 3.

Substrate	Specific Activity	
	μmol·mg protein^{-1}·min^{-1}	% control
ATP (control)	3.60 ± 0.05	100
GTP	0.04 ± 0.03	1
UTP	0.04 ± 0.02	1
CTP	0.59 ± 0.02	16
PPi	0.04 ± 0.04	1
ADP	0.09 ± 0.02	3
GDP	0.17 ± 0.04	5
UDP	0.06 ± 0.02	2
CDP	0.04 ± 0.01	1
IDP	0.07 ± 0.02	2

plasma membrane origin. Maximum ATPase activities 3.5 to 4.5 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ have routinely been obtained by this procedure.

To demonstrate further the homogeneity of the PM vesicles isolated by aqueous phase partitioning, U_3 vesicles were subjected to linear sucrose gradient centrifugation. As shown in Figure 1, the single peak of ATPase activity corresponded to the region of maximum protein concentration (A_{280}) at a sucrose density of 1.18 $g \cdot cc^{-1}$. This is at the high end of a range of values that is generally reported for plasma membranes (1.13–1.17 $g \cdot cc^{-1}$, 28).

The characteristics of the ATPase from the phase-purified PM were then determined. The PM ATPase had a high substrate specificity for ATP (Table II) with only CTP giving any significant activity compared to the control. The pH optimum was found to be about 6.5 (Fig. 2), and the activity showed a sharp decline in the region of normal plant cytoplasmic pH (approximately 7.1; 19, 21). The second peak at 5.3 was found even when the buffer system was varied and when molybdate was present and cannot be explained at this time. The ATPase had a requirement for Mg^{2+}, was unaffected by azide and nitrate, but was almost completely inhibited by 0.5 mM vanadate (Table III). The slight molybdenum inhibition may have been due to a nonspecific phosphatase associated with the membranes or in residual cytoplasm trapped within the vesicles during the initial disruption of the tissue. Figure 3 shows the temperature profile for the PM ATPase assay. There was a striking increase in ATPase activity between 25 and 30°C that may have reflected a lipid phase change from a gel to a liquid crystalline state that could have affected enzymic activity (11). Maximum activity was reached at 37°C. The reduced activity beyond 37°C was probably due to denaturation of the enzyme. These results correspond well with published values (24, 25).

Any ATPase present in right-side out vesicles which are sealed

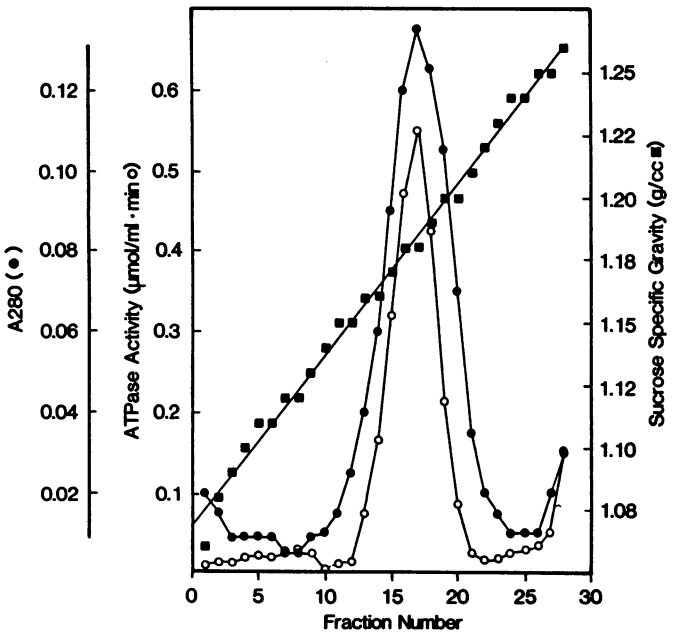

FIG. 1. Plot of protein (A_{280}, ●) and ATPase activity (○) after centrifugation of PM vesicles (isolated by phase partitioning) through a linear sucrose gradient. A_{280} was monitored as 0.5 ml fractions were collected. ATPase activity was assayed in 0.5 ml (20 min at 37°C) containing 5 mM $MgSO_4$, 50 mM KCl, 125 mM sucrose, 5 mM NaATP (pH 6.5), 0.1 mM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 30 mM MES/Tris (pH 6.5), and 0.0125% (w/v) Triton X-100. Sucrose concentration was determined refractometrically. This plot represents one of two identical centrifugation runs of two different extractions.

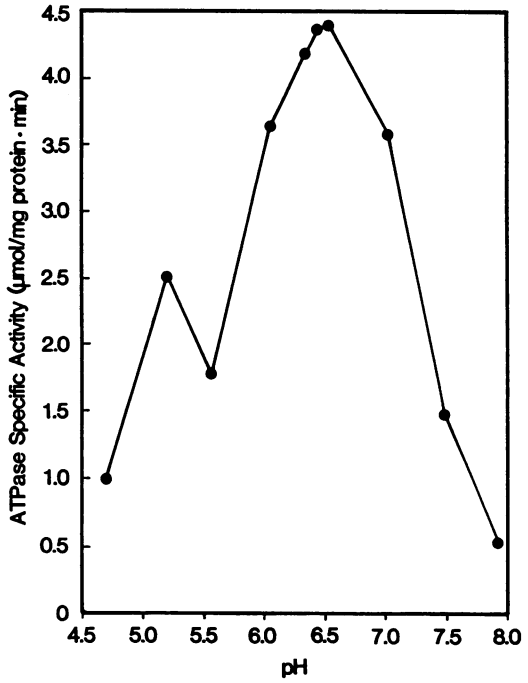

FIG. 2. Plot of ATPase activity *versus* pH. Half-ml samples containing 2 to 2.5 μg of PM protein were assayed as described in Figure 1. Each point is the mean of four different extractions. Buffers used were: Tris/acetate (pH 4.5–5.0), Mes/Tris (pH 5.5–6.5), and Hepes/Tris (pH 7.0–8.0).

Table III. *Effect of Ions and Inhibitors on U_3PM ATPase Activity*

ATPase activity was assayed as described in Figure 1. Where appropriate, the following salts were included in the assay: 5 mM $MgSO_4$, 50 mM KCl, 0.1 mM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.5 mM NaN_3, 50 mM $NaNO_3$, and 0.5 mM Na_3VO_4. Values represent the means ± SE; $n = 4$.

Ion	Specific Activity	
	$\mu mol \cdot mg\ protein^{-1} \cdot min^{-1}$	% control
Mg^{2+} + K^+ (control)	4.2 ± 0.20	100
Mg^{2+} ($-K^+$)	1.2 ± 0.20	29
K^+ ($-Mg^{2+}$)	0.2 ± 0.01	4
Azide	4.2 ± 0.20	100
Nitrate	4.2 ± 0.20	100
Molybdate	3.8 ± 0.20	90
Vanadate	0.1 ± 0.02	2

to the substrate, ATP, will be latent; *i.e.* increased activity occurs only when substrate is allowed to reach the hydrolytic site of the ATPase at the inner face of the PM. The percent activation of ATPase by Triton X-100 or other surfactants is usually taken to represent the amount of latent ATPase (9). Figure 4 shows PM ATPase activity of the U_3 in the presence of varying percentages of Triton X-100 (at 37°C). Higher concentrations of Triton X-100 were inhibitory. It is interesting to note that the activity actually dropped below the 0% Triton value at about 0.04% (w/v, T:p = 80).

Increasing lysolecithin concentrations had a very different effect on ATPase activity. ATPase activity appeared to approach a maximum value at lysolecithin concentrations lower than Triton X-100, then plateaued and did not decrease as the surfactant level was further increased. Thus, lysolecithin does not appear to inhibit the ATPase as Triton X-100 does at higher concentrations. The Triton inactivation of the PM ATPase at higher concentrations is reversible by simple dilution of the surfactant. PM vesicles incubated for 5 min in 0.04% (w/v, T:p

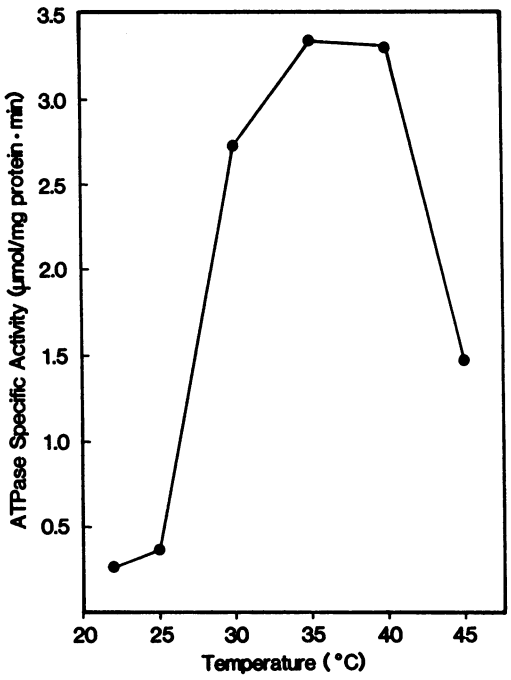

FIG. 3. Effect of temperature on PM ATPase activity in the presence of 0.0125% (w/v) Triton X-100. ATPase assays were run at the indicated temperature for 20 min as described in Figure 1. Values represent the mean of triplicate samples from one extraction.

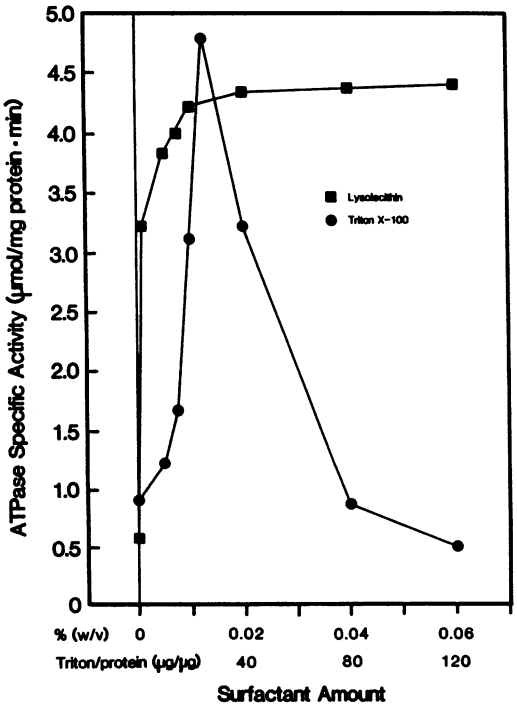

FIG. 4. Plot of ATPase activity in the presence of different concentrations of surfactants. Half-ml samples containing 2 to 2.5 μg of PM protein were assayed as described in Figure 1. Values for Triton X-100 (●) represent the mean of four different extractions. Values for lysolecithin (■) represent the mean of triplicate samples from one extraction.

= 80) Triton X-100 and then diluted to 0.0125% (w/v, T:p = 25) and assayed for ATPase activity showed less than a 20% loss of activity from a 0.0125% (w/v, T:p = 25) Triton control which had not been treated with the higher concentration of surfactant (data not shown).

To test further the sidedness and sealedness of the vesicles, we determined the effect of trypsin on the ATPase in the presence or absence of Triton X-100. If nearly all of the ATPase is found in sealed, right-side out vesicles, then trypsin should have little effect unless the vesicles are disrupted with surfactant. Table IV shows the effect of trypsin pretreatment on the PM ATPase activity. Trypsin alone, as well as trypsin plus trypsin inhibitor, had little effect on the ATPase activity. However, trypsin in the presence of 0.0125% (w/v, T:p = 25) Triton reduced the ATPase activity significantly, indicating access of the protease to the hydrolytic site of the ATPase. The reduction of ATPase activity in the presence of Triton and trypsin could be reversed by the presence of trypsin inhibitor. There appeared to be a slow loss of ATPase activity over time in the treatments that contained trypsin but no Triton X-100 (data not shown); however, the rate was much lower than when Triton was present.

To understand how the surfactants activated the PM ATPase, it was necessary to determine whether the ability of ATP to enter the vesicles was correlated with the ATPase activity at varying Triton to protein ratios. Therefore, a study was conducted to compare the effect of the T:p ratio on ATPase activity and on vesicle integrity, as measured by ESR, using identical conditions for both assays (Fig. 5). The ESR study required that the protein concentration be considerably higher than that used in the normal ATPase assay (2.12 mg/ml *versus* 0.005 mg/ml, respectively). To compensate for the increased ATPase activity resulting from the higher protein levels, the temperature was reduced to 22°C for the ATPase assays which resulted in significantly lower ATPase activity.

In the absence of Triton X-100, the vesicles were mostly sealed to TEPA, an ESR quenching molecule which is similar in both size and charge to ATP but which is not membrane permeable. As the ratio of Triton to protein increased, there was a steady decrease in the amount of sealed vesicles until, at a ratio of 0.1 (0.02% w/v), almost all the vesicles were open as determined by the quenched ESR signal. However, activation of the ATPase occurred only when the ratio exceeded 0.1 (0.02% w/v), with maximum activity being obtained at a ratio of 1 (0.2% w/v). This indicates that the apparent latency is not due solely to increased substrate availability, but to some other mechanism that involves a more direct interaction with the surfactant and

Table IV. *Effect of Trypsin on PM ATPase in the U_3 Fraction*

Treatments were run in 100 μl volumes for 5 min at 25°C and included PM vesicles (0.2 μg/μl PM protein), 125 mM sucrose, and 100 mM Tris/HCl (pH 8.0). Where appropriate, trypsin was used at a ratio of 2:1 (w/w, PM protein:trypsin), trypsin inhibitor at a ratio of 1.5:1 (w/w, inhibitor:trypsin), and Triton X-100 (0.0125% w/v). Treatments were stopped by adding inhibitor to the mixtures (that did not already contain inhibitor) and placed on ice. ATPase assays included 0.0125% (w/v) Triton X-100 as described in Figure 1 and "Materials and Methods." Values represent the means ± SE; $n = 2$.

Additions to Pretreatment Solutions	Specific Activity	
	μmol·mg protein^{-1}min^{-1}	*% control*
PM (Control)	4.4 ± 0.08	100
Trypsin	4.1 ± 0.06	93
Trypsin + Trypsin inhibitor	4.5 ± 0.01	102
Trypsin + Triton X-100	1.7 ± 0.60	39
Trypsin + Trypsin inhibitor + Triton X-100	4.3 ± 0.20	98

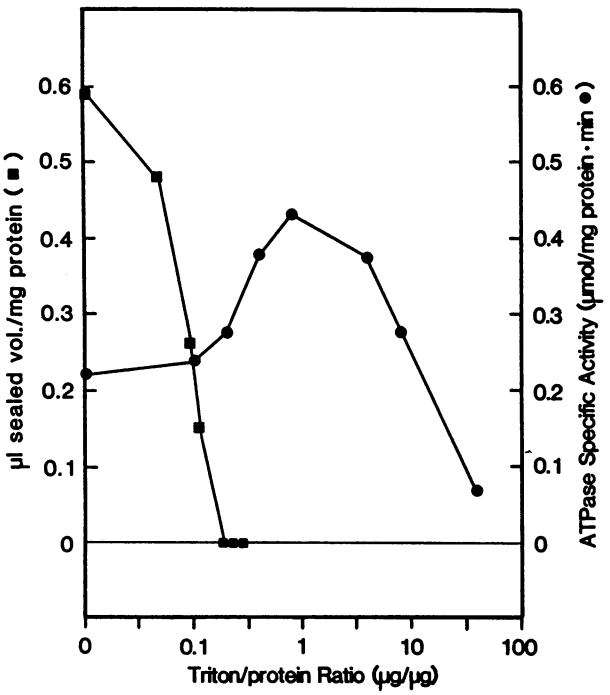

FIG. 5. Effect of Triton X-100 on sealed volume (■), assayed using ESR, and vanadate sensitive ATPase activity (●) of U_3 PM vesicles. Both assays were completed under identical conditions: 2.12 mg protein/ml, 22°C and varying amounts of Triton X-100.

the intrinsic protein.

Of interest here is that the percent of the Triton X-100 (0.1% w/v) that gave maximum ATPase activity in the smaller ESR volume (45 µl), but contained a high membrane concentration, was about 10 times greater than in the larger volumes (0.0125% in 500 µl) of the typical ATPase assays containing a more dilute membrane solution; the Triton to protein ratios are 1 and 25, respectively. Further studies (data not shown) confirmed that as the protein concentration was decreased, the Triton to protein ratio needed for maximum ATPase activity increased while the percent (w/v) decreased. The above results suggest that the amount of surfactant required to permeabilize the PM vesicles is different (less) than the amount needed to activate the intrinsic ATPase.

DISCUSSION

The oat root PM vesicles, prepared in this study by aqueous two-phase partitioning, are similar in purity and in the properties of the ATPase to those obtained by others (7, 9, 10, 23, 30) using the same materials and methods. However, they possess an unusually high maximum ATPase specific activity (about 4 $\mu mol \cdot mg^{-1} \cdot min^{-1}$). Low specific activities (approximately 0.6 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ or less) are normal for ATPases from PM vesicles isolated by both aqueous phase partitioning and density gradient partitioning (9, 25, 29). However, the high ATPase specific activity demonstrated in this study is not unprecedented. Surowy and Sussman (27), Hodges and Mills (7), and Anton and Spanswick (2) show elevated PM ATPase activities after density gradient enrichment. Only the purified and reconstituted PM ATPases of Serrano (23), Cocucci and Marrè (3), and Anton and Spanswick (2) show higher activities. It will be interesting to see whether the high specific activity of the ATPase described in this study is due to an unusually high level of ATPase in the oat roots, grown under our conditions, or whether it reflects decreased inactivation of the enzyme during preparation of the vesicles. Another unusual aspect of this ATPase preparation is the degree to which it is stimulated by K^+. In most previous investigations the maximum stimulation by K^+ was 2-fold (9, 24), while here it was closer to 4-fold.

The latency of the ATPase in PM vesicles, obtained by the aqueous two-phase partitioning, has been noted before (9, 10). In this study, over 80% of the U_3 ATPase was latent when assayed in the presence of Triton X-100 or lysolecithin. There was little significant difference in the maximum ATPase activity produced in the presence of the two surfactants; but there were differences in the concentration-activity curves. ATPase activity in the presence of lysolecithin approached a maximum similar to that of the Triton X-100 treated samples; however, the evidence suggests that maximum activity occurs at lower lysolecithin concentrations than Triton X-100. This is consistent with the idea that solubilization of membrane proteins by surfactants occurs at or near their critical micelle concentration (6). Higher concentrations of lysolecithin were without effect on ATPase activity where Triton X-100 inhibited. Similar results with Triton X-100 were obtained by Surowy and Sussman (27) and Larsson *et al.* (9) with oat roots; although, in the latter study it appeared that significantly higher surfactant concentrations (0.05% w/v) were required for maximum activity.

The effectiveness of surfactants has usually been ascribed to their ability to render the vesicles permeable to ATP (9), thus allowing access of the ATP to the ATP hydrolytic site within right-side out vesicles and permitting maximum activity. The sensitivity of ATP hydrolysis to a protease in the presence of surfactants is consistent with the notion that the PM vesicles were sealed to ATP and thus right-side out. The ESR data indicate that most of the vesicles were initially sealed at 22°C, but that addition of sufficient Triton X-100 completely destroyed their integrity. Surprisingly, this loss of vesicle integrity was not accompanied by any significant increase in ATPase activity. Even without Triton, TEPA does penetrate these 'sealed' vesicles to some extent, quenching the ESR signal, especially at 37°C (TL Lomax, RJ Melhorn, unpublished data), and may indicate that ATP also penetrates these vesicles under the same conditions; thus, to some extent they are leaky. The ESR results found at the lower temperature indicate, however, that maximum ATPase activity occurred only after vesicle integrity was completely destroyed and leakage not a factor. This suggests that the surfactant can act on the ATPase in a more direct manner to induce maximum activity.

The hydrophobic and hydrophilic environment around membrane proteins is important to their function (6, 8, 11, 22). Surfactants profoundly influence this environment by interacting with the lipids of the membrane as well as the hydrophobic domain of the protein. At low concentrations, surfactants bind to the vesicles, causing lysis and solubilization of mixed micelles containing surfactant, lipid, and protein. As the surfactant concentration is increased, the ATPase may be activated, either by a change in conformation or by an alteration of the insertion of the protein into the lipid bilayer. Alternatively, the surfactant may cause the loss of an inhibitory subunit. Both possibilities have been proposed for the surfactant activation of the Na^+, K^+-ATPase (8). With even higher levels of Triton X-100, the ATPase may become delipidated or unstable, and thus inactivated, while lysolecithin continues to provide an appropriate lipid environment. The reversal of the Triton inactivation of the PM ATPase suggests that this surfactant is either not completely delipidating the enzyme or is a satisfactory lipid substitute at an appropriate concentration.

In conclusion, we have demonstrated that the latency of the PM Mg^{2+}, K^+-ATPase is not merely a function of increasing substrate availability by opening sealed, right-side out vesicles, but can also be the result of a direct activation of the enzyme by surfactants. Elucidating the nature of that activation may give

exciting insights into the *in vivo* regulatory mechanisms for this physiologically important enzyme.

Acknowledgment—We would like to thank Dr. Rolf J. Mehlhorn for his assistance in the ESR determinations.

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