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Plasma Membrane ATPase Activity following Reversible and Irreversible Freezing Injury[1]

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ABSTRACT

Plasma membrane ATPase has been proposed as a site of functional alteration during early stages of freezing injury. To test this, plasma membrane was purified from *Solanum* leaflets by a single step partitioning of microsomes in a dextran-polyethylene glycol two phase system. Addition of lysolecithin in the ATPase assay produced up to 10-fold increase in ATPase activity. ATPase activity was specific for ATP with a K_m around 0.4 millimolar. Presence of the ATPase enzyme was identified by immunoblotting with oat ATPase antibodies. Using the phase partitioning method, plasma membrane was isolated from *Solanum commersonii* leaflets which had four different degrees of freezing damage, namely, slight (reversible), partial (partially reversible), substantial and total (irreversible). With slight (reversible) damage the plasma membrane ATPase specific activity increased 1.5- to 2-fold and its K_m was decreased by about 3-fold, whereas the specific activity of cytochrome c reductase and cytochrome c oxidase in the microsomes were not different from the control. However, with substantial (lethal, irreversible) damage, there was a loss of membrane protein, decrease in plasma membrane ATPase specific activity and decrease in K_m, while cytochrome c oxidase and cytochrome c reductase were unaffected. These results support the hypothesis that plasma membrane ATPase is altered by slight freeze-thaw stress.

As early as 1912 (11), the plasma membrane was recognized as a site of alteration due to freezing injury. However, the nature of injury at a more fundamental level has been investigated only in recent years (3, 7, 13–19, 22–25, 29). In nature, herbaceous plants experience slow cooling during a freezing event (at a rate of about 1°C/h) (7, 22, 24). Visual symptoms following thaw include loss of turgor and a water-soaked appearance of the tissue (7, 14). Associated with these symptoms is an increase in efflux of ions and organic solutes (15). These symptoms have led some investigators to assume that freezing injury results in membrane rupture and a complete loss of membrane semipermeability (25). Contrary to this view, Palta and coworkers (13–18) have demonstrated that incipient freezing injury results in an alteration of K^+-transport properties of the membrane and illustrated that freezing injury was reversible in potato leaves and onion bulb scale tissue. These studies showed that after incipient freezing injury the leakage of specific ions increased, and there was no change in the *in vivo* membrane permeability to water, urea, and methyl urea. Reversibility of the injury was illustrated by the disappearance of water soaking and reuptake of leaked ions back into the cell. Based on their results, Palta and Li (17, 18) proposed alteration of plasma membrane ATPase as an early event during incipient freezing injury.

Recent studies by three research groups (3, 19, 29) have provided data in support of the hypothesis by Palta and coworkers. In our laboratory, we have found no change in photosynthetic and respiratory activity, while ion efflux was more than doubled compared to control following reversible freeze-thaw injury in the leaf tissue of two potato species (22, 24). The ultrastructural observation of these cells revealed no abnormalities (13, 17). Taken all together, the results from these studies on potato and our earlier studies on onion tissue demonstrate that plasma membrane functions in these cells are altered following a reversible injury. ATPase was suggested as the target for alteration since ion uptake is driven by ATPase either directly or indirectly. However, the exact nature of this alteration to ATPase has not been investigated.

To understand the mechanism of freezing injury it is important to investigate the nature of alteration to plasma membrane ATPase in tissue with subtle as well as stepwise progressively increased freezing injuries. A number of studies have utilized severely injured (lethal injury) tissue for investigation of freezing injury mechanisms. These studies have missed out important information on the early events following injury.

Therefore, a systematic study of changes in plasma membrane ATPase following stepwise progressive freezing injury is needed. Potato species are highly suited for this purpose because of the availability of: (a) related species with considerable differences in freeze stress tolerance and cold acclimation ability, and (b) abundant background information on freeze stress responses of these species (8, 13, 17, 22–24). The present study was designed to investigate changes in the specific activity and K_m of the plasma membrane ATPase following slight (nonlethal, reversible) as well as severe (lethal, irreversible) injury.

MATERIALS AND METHODS

Plants were grown from clonally propagated plantlets obtained from micropropagated cultures on MS media (12). Leaf tissue of a wild potato species *Solanum commersonii* (PI 472834) and cultivated species *Solanum tuberosum* L. cv Red Pontiac, were used. Rooted plants were transferred to 20 cm

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diameter pots filled with 1:1 (w/w) milled sphagnum and perlite (Jiffy mix, JPA, West Chicago, IL) and grown for 6 to 8 weeks in growth chambers 1.3 × 2.5 m (l × b) at 20°C/15°C (light/dark) with 14 h of light period with 400 μmol m^{-2} s^{-1} photosynthetic photon flux densities (400–700 nm) from cool-white fluorescent lamps. Plants were irrigated four times daily with half-strength Hoagland nutrient solution supplemented with an additional 2.5 mM $Ca(NO_3)_2$. The nutrient solution was supplied in excess. RH was neither controlled nor measured.

Preparation and Characterization of the Plasma Membrane

Solanum commersonii is a cold-tolerant (LT_{50} of about −5 and −10°C before and after acclimation, respectively) and *Solanum tuberosum* is a cold-sensitive (LT_{50} of about −2.5°C and unable to acclimate) potato species (8, 13, 22–24). All operations were carried out between 0 and 4°C. Fully expanded leaflets were excised from two plants which were 6 to 8 weeks old. The lower five to six leaves were not used as these start senescing after about 2 months. About 50 g of excised leaflets were ground in a mortar and pestle in about 75 mL of homogenizing buffer (28) (0.25 M sucrose, 75 mM Mops-NaOH, 5 mM EGTA, 1 mM PMSF, 2 mM salicyl hydroxamic acid, 2.5 mM sodium bisulfite, 1.5% PVP of mol wt 24000, 0.5% BSA, 10 μg/mL BHT[2] [pH 7.6]) and filtered through four layers of cheese cloth. The filtrate was centrifuged at 8,000*g* for 20 min. The supernatant was pelleted at 48,000*g* for 90 min. This pellet was suspended in 0.25 M sucrose in 10 mM K-phosphate buffer (pH 7.3) and partitioned in a two phase system with a final concentration of 5.6% (w/w) each of dextran T-500 and polyethylene glycol, mol wt ~3500 (PEG) with 0.25 M sucrose, 10 mM K-phosphate buffer (pH 7.3) and varying concentrations of NaCl. The phase separation was facilitated by centrifuging at 2,500*g* for 10 min. The UP was aspirated. The UP and LP were diluted about 10-fold with phase diluting buffer (0.25 M sucrose, 5 mM Mops-NaOH, 1 mM EGTA, 10 mM KCl, 0.2 mM PMSF, 10 μg/mL BHT, and 1 mM DTT [pH 7.3]), and centrifuged at 48,000*g* for 20 min to pellet the membrane fractions and also to get rid of the polymers (PEG and dextran). The membrane pellets were suspended in a minimal amount of phase diluting buffer and stored at −80°C.

The membrane ATPase was assayed by the colorimetric determination of Pi released from ATP (27). The assay was carried out at 30°C in a total volume of 0.5 mL for 30 min. The reaction was started by the addition of the membrane fraction to a mixture containing 5 mM ATP, 5 mM $MgCl_2$, 5 mM phospho*enol*pyruvate, and 10 μL pyruvate kinase (for regeneration of ATP), 10 mM Pipes, 100 mM KNO_3 (to inhibit the vacuolar ATPase), 5 mM NaN_3 (to inhibit the mitochondrial ATPase), 0.1 mM ammonium molybdate (to inhibit the nonspecific phosphatases). The reaction was stopped by adding 0.1 mL of 50% TCA.

[2] Abbreviations: BHT, butylated hydroxytoluene; CCO, Cyt *c* oxidase; Mic, Microsomes; NADH-CCR or CCR, NADH-dependent Cyt *c* reductase; UP, upper phase; LP, lower phase + interface; PK, pyruvate kinase; LDH, lactic dehydrogenase.

The K_m of ATPase was determined by a coupled assay based on the method of Auffret and Hanke (1). The ATPase activity was coupled to PK and LDH activities. The assay was performed at 30°C in a total volume of 1.5 mL and contained in addition to all ingredients used in the colorimetric assay, LDH (10 μL) and NADH (250 μM) and the required amount of the membrane. The reaction was started by the addition of different concentrations of ATP, and the decrease in A_{340} with time due to the oxidation of NADH was recorded. The concentration of Mg·ATP was calculated according to Storer and Cornish-Bowden (26). This assay has an advantage over the colorimetric assay for determination of K_m since it allows recording of the enzyme activity as a function of time. The kinetic parameters were determined by using the program PENNZYME (9).

The assay of CCO and NADH-CCR was according to the method of Hodges and Leonard (4). Preparation of vanadate, Chl estimation, and protein estimation were according to the procedure of Surowy and Sussman (27), Wintermans and DeMots (30), and Markwell *et al.* (10), respectively.

Discontinuous SDS-PAGE was carried out according to Laemmli (6). Stacking and running gels were 4 and 10%, respectively. Gels were stained with Coomassie blue. Immunoblotting and visualization of the bands were according to Surowy and Sussman (27). Following electrophoresis the SDS gels were electroblotted on to nitrocellulose in transelectroblot cells for 3 h. The nitrocellulose was blocked overnight in BSA-blocking buffer, then incubated with the antibody (1:500 diluted) for 1 h and washed by four changes of the buffer every 15 min. This was followed by incubation with alkaline phosphatase-conjugated double antibody (1:500 diluted) and washing with four changes of the buffer every 15 min. The bands were visualized by reaction with substrates *p*-nitroblue tetrazolium (NBT) and 5-bromo, 4-chloro, 3-indoyl phosphate (BCIP).

Freeze-Thaw Treatment

Leaf tissue of *S. commersonii* was used for these studies. For each experiment, one to three plants of the same age and grown from the same stock under identical conditions were used. Fully expanded leaflets were excised between 8:00 and 9:00 AM and kept on ice. Random samples from the pooled leaves were used for control and freezing treatments. For the freezing treatment, leaflets were introduced into 50 to 100 tubes (2 × 19 cm with two leaflets in each tube) and inserted into a temperature controlled cooling bath set at 0°C. The temperature was lowered at a rate of 0.5°C/30 min with ice nucleation at −0.5°C. For control samples the temperature was maintained at 0°C. Temperature of the leaflets was monitored with copper-constantan thermocouples placed on a few leaflets. After reaching the desired temperature, the samples were held at that temperature for 15 min and then thawed on ice overnight.

Determination of Freezing Injury

After thawing, samples were taken out of ice, equilibrated for about 2 h in a cold room at 4°C, and visually examined for the extent of water soaking and turgidity. Water soaking

was rated on a 0 to 100% scale and turgidity on a A–C scale. Leaflets were separated into four categories of damages: (a) slight damage: 10–25% water soaking and turgidity A, majority of the leaflets frozen to −3° were in this category; (b) partial damage: 25 to 50% water soaking and turgidity AB, majority of the leaflets frozen to −4.5°C were in this turgidity (c) substantial damage: 70 to 90% water soaking and turgidity C (totally lost); (d) total damage: 100% water soaking and turgidity C. Leaflets in the last two categories were obtained from leaflets frozen to −5 and −6°C. The control had no water soaking and was fully turgid.

After separation into the above categories at least three separate samples were saved to determine the extent of damage by the measurement of ion leakage (15). The rest of the tissue was used for the isolation of plasma membrane according to the procedure described above. Excised leaflets which were kept at 0°C taken from the same plants at the same time (before freezing treatment) served as control.

Dextran T-500 was obtained from Pharmacia Fine Chemicals. PVP was from Aldrich. Reagents for electrophoresis were from Bio-rad Labs. All other chemicals were from Sigma Chemical Co.

RESULTS

Purification and Characterization of Plasma Membrane

The purification procedure of Kjellbom and Larsson (5), gave membrane preparations with a relatively high plasma membrane ATPase activity in the UP obtained after the first partitioning of microsomes (Table I). However, there was also a considerable amount of NADH-CCR activity, CCO activity and Chl content in the UP indicating contamination by ER, mitochondrial, and chloroplast membranes, respectively (Table I). We sought to modify the procedure to obtain pure plasma membrane by only one step of partitioning. Buffer compositions and salt concentrations were modified suitably. The results are shown in Tables II and III. For leaves of both *Solanum* species, UP membranes prepared in the presence of 30 mM NaCl were rich in plasma membrane ATPase activity and had only 0 to 5% contamination from mitochondrial, chloroplast, and ER membranes (indicated by the CCO activity, Chl content, and NADH-CCR activity, respectively). There was a higher contamination from these organelle membranes in the preparation obtained with 10 and 20 mM NaCl in the phase mixture. The amount of CCO and NADH-CCR activities and Chl in the UP decreased with increase in concentration of NaCl. In separate samples, UDPase activities (2) were measured in the plasma membrane fraction to examine the presence of Golgi membranes. This activity was 4.6 and 4.9% of that in microsomes in *S. tuberosum* and *S. commersonii*, respectively. Hence, partitioning in the presence of 30 mM NaCl was the best for plasma membrane preparation from both species and was employed for all further work.

The procedure outlined above was repeated a number of times, and a range of values from different batches of plants were obtained. The specific activity of ATPase in the microsomes was 0.054 to 0.13, μmol of Pi released mg protein^{-1} min^{-1}. The specific activity in the UP was 0.13 to 0.73 μmol mg protein^{-1} min^{-1}. Protein in UP was 4 to 10% of the microsomes. ATPase activity in the UP was 26 to 55% of the microsomes. CCO, NADH-CCR, and Chl content in UP was always less than 5% of the microsomes. It is important to note that variation in the specific activity of plasma membrane ATPase in the UP in different samples taken randomly from a pool of excised leaflets was always less than 10%. Variation in the specific activity of ATPase and the amount of plasma membrane protein in isolations on different days comes from differences in the amount of starting material, different batches of plant material and varying protein to polymer ratios during partitioning.

In the presence of lysolecithin, ATPase activity was enhanced to a greater extent in the UP membranes compared to the enhancement seen in the microsomes and LP (Tables II and III). Lysolecithin produced a maximum of 2-fold activation in microsomes and LP whereas in UP it ranged from 4- to 10-fold in different preparations.

In the absence of KNO_3 in the assay mixture, the total ATPase activity was increased by about 20 to 30% in the microsomes and LP (Table IV). There was no increase of ATPase activity in UP membranes. Similarly, in the absence of azide, the activity increased in the microsomes and LP whereas no increase was detected in the UP. There was no increased activity in the absence of molybdate in any of the fractions of *S. commersonii*. The molybdate sensitive ATPase activity was present in the microsomes and LP but not in UP in *S. tuberosum*. In the presence of 100 μM vanadate (a specific inhibitor of plasma membrane ATPase), there was 70 to 80% reduction in the ATPase activity in the UP (Table IV). These data show a lack of contamination by tonoplast ATPase, mitochondrial ATPase, and phosphatases and also show the enriched plasma membrane ATPase in the UP.

The substrate specificity of ATPase is shown in Table V. In the microsomes there was extensive hydrolysis of ADP in addition to ATP and also, to a certain extent, hydrolysis of AMP. The specificity for ADP and AMP was much reduced in the UP compared to that in the microsomes. The extent of hydrolysis of other nucleoside-triphosphates tested was very little.

The initial velocity of the reaction was checked with different concentrations of ATP ranging from 0.01 to 5 mM. The rates observed did not change even when the concentrations of PK and LDH were doubled, showing that the intermediate indicator reactions were not rate limiting. The kinetic param-

Table I. *Partitioning of S. commersonii Microsomes Obtained from the Leaf Tissue by the Method of Kjellbom and Larsson (5)*

Standard assays described under "Materials and Methods" were used to measure the activities of plasma membrane ATPase (with the inhibitors and the detergent lysolecithin; at 30°C.

Fraction	Protein	ATPase Activity		Total Activity		Chl
		SA[a]	%	CCO	CCR	
	mg				%	
Microsome	48	0.11	100	100	100	100
UP	21	0.18	72	25	25	82
LP	6	0.29	33	28	23	26

[a] SA expressed in μmol Pi mg^{-1} min^{-1}.

Table II. *Effect of NaCl on the Partitioning of S. tuberosum Plasma Membrane in a Two Phase Polymer System*

The values given are from a representative experiment and are averages of duplicates. The values given for the microsomes represent the amount used for partitioning with each concentration of NaCl. Typically, for assays, the amount of protein used was: 100 to 200 μg of microsomes and LP, and 25 to 50 μg of UP. LPC, Lysophosphotidylcholine (Lysolecithin); SA, specific activity. Assays were as described under "Materials and Methods." Other abbreviations as in the abbreviation footnote.

Fraction	Protein	ATPase Activity				Total Activity		Chl
		SA[a]	%	SA without LPC	Activation with LPC	CCO	CCR	
	mg				*-fold SA*		%	
Microsomes	37	0.13	100	0.086	1.6	100	100	100
10 mM NaCl								
UP	7.1	0.37	55	0.11	3.3	8.3	27	28
LP	7.0	0.20	29	0.10	2.0	41	44	58
20 mM NaCl								
UP	4.7	0.40	39	0.19	2.1	2.1	17	10
LP	11	0.17	39	0.10	1.7	63	57	82
30 mM NaCl								
UP	3.2	0.73	49	0.18	4.1	0.73	2.5	4.3
LP	9.8	0.19	39	0.13	1.5	41	66	71

[a] SA expressed in μmol Pi mg^{-1} min^{-1}.

Table III. *Effect of NaCl on the Partitioning of S. commersonii Plasma Membrane in a Two Phase Polymer System*

Assay was as described under "Materials and Methods" and was in the presence of lysolecithin. Abbreviations same as in the abbreviation footnote.

Fraction	Protein	ATPase Activity				Total Activity		Chl
		SA[a]	(%)	SA without LPC	Activation with LPC	CCO	CCR	
	mg				*-fold SA*		%	
Microsomes	37	0.061	100	0.057	1.1	100	100	100
10 mM NaCl								
UP	8.4	0.13	48	0.048	2.7	13	35	14
LP	16	0.05	35	0.017	2.9	37	38	52
20 mM NaCl								
UP	5.0	0.16	35	0.054	3.0	7	4	4
LP	19	0.041	33	0.029	1.4	29	36	77
30 mM NaCl								
UP	3.4	0.26	38	0.064	4.0	2.6	0	2
LP	19					51	74	

[a] SA expressed in μmol Pi mg^{-1} min^{-1}.

eters obtained for *S. commersonii* were K_m: 0.39 ± 0.1 mM and V_{max}; 0.29 ± 0.01 μmol mg^{-1} min^{-1} and for *S. tuberosum* K_m: 0.30 ± 0.01 mM and V_{max}: 0.56 ± 0.00006 μmol mg^{-1} min^{-1}.

The SDS-PAGE pattern and the immunoblots of the gel are shown in Figure 1, A and B. UP was enriched in the 100 kD band. Other specific bands were enriched in the LP (data not shown). Immunoblot showed the specific staining with oat-ATPase antibody in the microsomes and UP fractions (Fig. 1B). Similar results were obtained with the membrane preparations from *S. commersonii* (data not shown). Cross-reactivity between oat and potato root ATPase has been earlier demonstrated (27). Electroblots did not show any bands when preimmune sera was used instead of the antibody (data not shown).

Effect of Freezing Injury

Loss of turgor, increase in extent of visual water soaking and enhanced ion efflux easily separated the injured tissue into four categories as slight, partial, substantial, and total damage (Table VI). Following slight damage (totally reversible) there was a notable increase (1.5- to 2-fold) in the specific activity of ATPase in both the UP and microsomal membrane fractions compared to the paired controls. The amounts of protein recovered in the UP did not change markedly compared to control (Table VI). The K_m of ATPase for ATP was 0.13 ± 0.015 mM compared to 0.32 ± 0.047 mM in the control (Table VII), a 2.5-fold change in this kinetic parameter. However, the specific activity of CCO and NADH-CCR (enzymes of mitochondria and ER, respectively) was the same

Table IV. *ATPase Activity in the Presence and Absence of Different Inhibitors*

The values are averages ± SD of at least three different experiments. The values are calculated with respect to the standard assay performed with the same samples using the procedure described under "Materials and Methods." For-KNO_3 assays, KCl was included in the assay mixture. The values for assays in the presence of vanadate are from a single experiment and are averages of duplicates. Assay was as described under "Materials and Methods" and was in presence of lysolecithin. Abbreviations same as in the abbreviation footnote.

Inhibitor (+ present; − absent)	PM ATPase Activity					
	S. tuberosum			*S. commersonii*		
	Microsomes	UP	LP	Microsomes	UP	LP
	% ± *SD*					
$-KNO_3$	115 ± 8	102 ± 1	105 ± 1	128 ± 22	100	128 ± 23
$-NaN_3$	130 ± 10	90 ± 0.6	126 ± 7	144 ± 24	100	115 ± 9
−Molybdate	116 ± 12	100	123 ± 7	100	100	100
+Vanadate (100 μM)	55	27	38	59	22	24

Table V. *Substrate Specificity of ATPase*

Concentration of substrates used was 5 mM. While studying the activity toward ADP, the ATP regenerating system was omitted from the assay mixture. A control of ATP as substrate without the ATP regenerating system was also run in parallel. Activities are averages of duplicates ± SD. Assay was as described in "Materials and Methods" and was in presence of lysolecithin.

Substrate	PM ATPase Activity			
	Microsomes		Upper phase	
	S. tuberosum	*S. commersonii*	*S. tuberosum*	*S. commersonii*
	%			
ATP	100	100	100	100
ADP	158 ± 8	147 ± 18	20	7 ± 6
AMP	66 ± 15	77 ± 11	27	24 ± 12
CTP	48 ± 16	14 ± 19	0	4 ± 1
GTP	36 ± 2	59 ± 40	0	6 ± 2
ITP	47 ± 9	38 ± 33	0	5 ± 0.5
UTP	7 ± 11	42 ± 16	0	4 ± 3

in the microsomes of both the control and slightly damaged samples (Table VI).

Following partial damage, the specific activity of ATPase either decreased or was similar to the control, depending on the particular experiment. However, as in slightly damaged leaves, there was no effect on either the microsome or plasma membrane protein recovered (Table VI). The K_m for ATP in plasma membrane decreased from 0.53 ± 0.11 mM in the control to 0.069 ± 0.011 mM following partial damage (Table VII) which is an 8-fold change in this kinetic parameter. There was again no difference in the specific activity of CCO and NADH-CCR in the microsomes of partially damaged leaflets compared to control.

After substantial damage (irreversible) the specific activity of ATPase was to to four times lower than the control (Table VI). There was also about a 50% decrease in the amount of microsomal protein. However, the amount of plasma membrane protein (as a percentage of microsomes) did not decrease. The K_m for ATP decreased from 0.23 ± 0.052 mM to 0.017 ± 0.0068 mM, which reflects a 14-fold change in this kinetic parameter. Again, there was no change in the specific activity of CCO and NADH-CCR activities compared to that of the control.

Following total damage, the specific activity of ATPase decreased greatly and was barely detectable in some samples (Table VI). In addition to a 50 to 60% reduction in the amount of microsomal protein recovered, there was also a reduction in the plasma membrane protein (as a fraction of the recovered microsomes) by about 50%. The reduction in CCO activity was 38% and NADH-CCR activity was not affected.

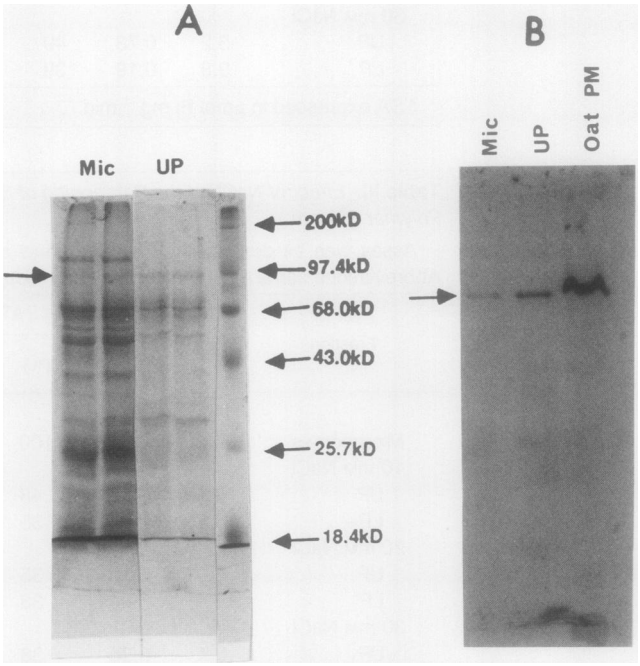

Figure 1. SDS-PAGE and immunoblots of *S. tuberosum* membrane fractions. A, SDS-PAGE of microsome (Mic) and upper phase (UP). Fifty μg of total protein were loaded in each lane. Molecular mass markers and their molecular masses are shown on the right. Arrow on the left indicates the location of the 100 kD band. B, immunoblot of microsome (Mic) and upper phase (UP). Immunoblot of oat plasma membrane (PM) is also shown for comparison.

Table VI. *Effects of Various Degrees of Freeze-Thaw Stress in S. commersonii Leaflets*

Standard assay conditions were used as described in "Materials and Methods" and Table I. The values are from two representative experiments.

Category of Damage	Visual Water Soaking Area	Turgidity	Ion Leakage[a]	Microsomal Protein		PM Protein		SA of ATPase[b]		CCR (SA)[c]	CCO (SA)[c]
				Leaves	% of Control	% of Mic.	% of Control	Mic.	PM		
	%		% of total	mg/g							
Slight	10–20	A	14 ± 6 (9 ± 2)[d]	0.95 (0.99)	96	6.8 (7.3)	93	0.13 (0.06)	0.51 (0.27)	22 (21)	3.7 (3.6)
	10–20	A	27 ± 6 (12 ± 3)	1.1 (0.96)	115	4.7 (5.1)	92	0.12 (0.06)	0.67 (0.31)		
Partial	25–50	AB	50 ± 8 (9 ± 2)	1.1 (1.1)	100	8.4 (8.1)	104	0.13 (0.13)	0.22 (0.45)	30 (36)	3.9 (2.7)
	25–50	AB	35 ± 7 (12 ± 3)	1.1 (1.0)	110	7.0 (8.0)	88	0.06 (0.12)	0.59 (0.59)		
Substantial	70–90	BC		0.34 (0.64)	53	10.8 (8.8)	118	0.013 (0.05)	0.09 (0.38)	57 (58)	1.3 (1.2)
	70–90	BC	76 ± 9 (12 ± 3)	0.58 (0.96)	60	8.8 (9.0)	89	0.054 (0.12)	0.4 (0.59)		
Total	100	C	85 ± 5 (15 ± 3)	0.21 (0.64)	33	4.4 (8.8)	50	ND[e]	ND	36 (58)	1.4 (1.1)
	100	C		0.54 (0.96)	56	2.8 (5.1)	55	0.023 (0.12)	0.16 (0.59)		

[a] Ion leakage values are a mean of 3–5 determinations ± SD. [b] SA of ATPase: μmol Pi released mg^{-1} protein min^{-1} (averages of duplicate determinations). [c] SA of CCO, CCR: assay carried out in microsomes, nmol Cyt *c* oxidized or reduced mg^{-1} protein min^{-1} (averages of at least five determinations). [d] The values in parenthesis are those of the corresponding control tissue (sample from same lot held at 0–4°C). [e] ND, Not detectable.

Table VII. *K_m and V_{max} of PM ATPase following a Freeze-Thaw Stress in S. commersonii Leaflets*

The values represent the best fit obtained by using the program PENNZYME. The values in parentheses are those of the corresponding nonfrozen control leaf tissue. For assays with paired control and injured samples similar amounts of membrane proteins were used.

Extent of damage	K_m	V_{max}
	mM	nmol min^{-1}
Slight	0.13 ± 0.015 (0.32 ± 0.047)	3.9 ± 0.16 (2.5 ± 0.11)
Partial	0.069 ± 0.011 (0.53 ± 0.11)	1.3 ± 0.08 (3.1 ± 0.22)
Substantial	0.017 ± 0.0068 (0.23 ± 0.052)	0.45 ± 0.032 (2.2 ± 0.15)

The SDS-PAGE profile of microsomes and plasma membrane before and after the four categories of freezing damage is shown in Figure 2. The protein pattern of microsomes (lanes 8–12) and plasma membrane (lanes 2–6) from control samples look very similar to injured samples. The intensity of 100 kD band (indicated by the arrow on the left in Fig. 2) is reduced with increased damage. It is interesting to note that though there was no ATPase activity in the totally damaged sample, the 100 kD band is clearly visible. The 100 kD band from totally damaged tissue was reactive with oat ATPase antibodies (data not shown).

DISCUSSION

Purity of the Plasma Membrane

The single-step phase partitioning procedure used in this study yielded enriched plasma membrane preparations from the leaves of two *Solanum* species (Tables II–IV). The recovery of marker enzymes and Chl content in UP + LP compared to microsomes after partitioning ranged from 41 to 80% (Tables II and III). This is similar to other reports (5). The K_m and substrate specificities were similar to those reported from other systems (21). Up to fourfold to tenfold activation of ATPase obtained in the presence of lysolecithin (Tables II and III) may be due to its permeabilizing effect on the membrane and also due to direct effects on the enzyme (3, 20, 28, 29).

Increase in Specific Activity of ATPase following a Reversible Freezing Injury

At this level of injury, the water soaking disappears with time. This recovery requires an uptake of leaked ions (which

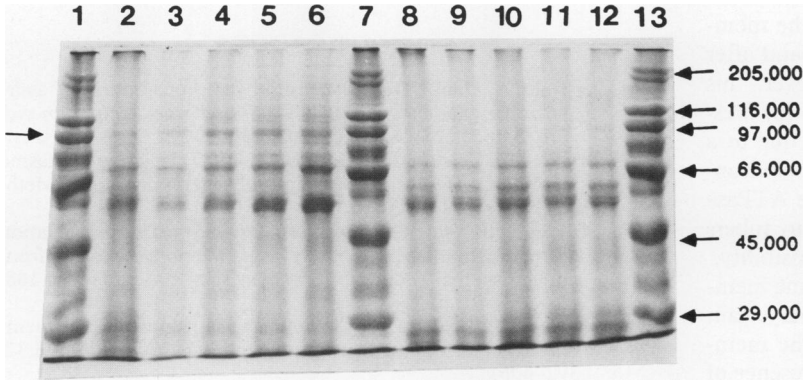

Figure 2. SDS-PAGE pattern of *S. commersonii* membrane fractions after freezing injury. Lanes 1, 7, and 13 are mol wt markers. Their mol wt are pointed on the right. Lanes 2 to 6 are, in order: PM from totally damaged, substantially damaged, partially damaged, slightly damaged, and control. Lanes 8 to 12 are, in order: microsomes from totally damaged, substantially damaged, partially damaged, slightly damaged, and control. Fifty μg of protein were loaded in each lane. Arrow on the left points the location the 100 kD band.

is 50–100% over the control) back into the cell (13). The increased ATPase activity observed following slight injury may help in this recovery process by pumping leaked ions back into the cell. Water then moves osmotically and the water soaked condition disappears (13, 14, 16).

Electron microscopic observations showed normal organelle ultrastructure following reversible injury in the leaf tissue of the potato species used in this study (13). We also know from other studies in our laboratory that tissue respiration and photosynthesis rates are unaffected in early stages of injury in the same species (22) indicating a higher sensitivity of plasma membrane functions to freeze-thaw stress relative to those of mitochondria and chloroplasts. This is also supported by our enzymic studies where the specific activity of CCO and NADH-CCR were unaffected (Table VI). Interestingly, a nearly twofold increase in specific activity of ATPase following 'sublethal' freezing injury was also observed in pine needles (3). It is difficult to assess the significance of this observation without detailed information on the post-thaw behavior of the tissue used in that study.

The increase in specific activity of ATPase following slight injury could be either due to (a) decrease in K_m or (b) increase in the amount of ATPase assembled into the membrane. The K_m decreased, indicating a 2.5-fold change in this kinetic parameter. It is important to note that the specific activity of ATPase increased not only in the purified plasma membrane fraction but also in the microsomal fraction (Table VI). This indicated that the observed increase in ATPase activity was not due to any selective perturbation of the stressed membranes during the partitioning of the membrane fraction in the two phase partitioning system.

The mechanism by which increase in ATPase activity is brought about needs to be investigated. Whether the increase in specific activity of ATPase results in an increased efficiency of ATPase functions needs to be examined. Among the different possibilities for an increased efficiency are: operation of more favorable H^+-gradients, $\Delta\psi$, and ΔpH; better coupling of ATPase activity to transport; and more efficient functioning even under a limited ATP supply.

Progressive Decrease in Specific Activity of ATPase following Irreversible Freezing Injury

Following substantial damage (irreversible, lethal injury), there was a progressive reduction in specific activity of ATPase (Table VI). There was also about a 13-fold decrease in K_m (Table VII). These could occur by a combination of different events such as: (a) loss of ATPase molecules from the membrane; a marked reduced intensity of the ATPase band after substantial damage suggests a possible loss; however, this needs confirmation by more quantitative tests; and (b) presence of ATPase molecules rendered nonfunctional due to a conformational change and/or an altered lipid microenvironment; an increased inhibition of plasma membrane ATPase activity by DCCD observed in *Helianthus tuberosus* tubers after lethal freezing injury (29) also points to this possibility.

Following total damage the specific activity of plasma membrane ATPase was greatly reduced (Table VI). This may again be due to a combination of loss of ATPase from the membrane and nonfunctional ATPase molecules. The presence of an ATPase band of reduced intensity at total damage (Fig. 2) (ATPase activity was not detected in this sample) supports these possibilities. The K_m was not measured because of very low activity of ATPase in the sample.

As the tissue is damaged severely (substantial and total) there will be a cascade of secondary biochemical effects which could lead to deterioration of the tissue. Among the secondary effects is a general loss of membrane/membrane protein (substantial damage, Table VI), followed by either a preferential loss of plasma membrane/plasma membrane protein or a change in surface properties of the plasma membrane (total damage, Table VI).

Mechanism of Freezing Injury

The mechanism of freezing injury is not clearly understood. Cooling rates and the time of initiation of ice formation in the tissue are known to influence the injury produced (7, 22, 24). The cooling rates used in the present study were close to what usually occurs in nature for herbaceous plants (7, 22, 24). Using a realistic freeze-thaw protocol we have documented that freezing injury is subtle and progressive rather than a cataclysmic event (13–18, 22, 24). In these studies no change in photosynthetic and respiration status (22, 24) and membrane ultrastructure (13) were detected following reversible freezing injury. In the present study, at a similar level of injury, no effect on CCO and NADH-CCR activities was found. These results support the hypothesis that plasma membrane ATPase is one of the initial sites of alteration following injury. These alterations include an increase in specific activity following reversible injury and progressive decrease in specific activity following a stepwise increase in the extent of irreversible freezing injury. Furthermore, our results clearly show a decrease in K_m of ATPase following freeze-thaw stress. An understanding of the molecular aspects of these alterations should provide an insight into the process of initiation of freezing injury in herbaceous plant species.

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