Pergamon

0031-9422(95)00707-S

Phytochemistry, Vol. 41, No. 3, pp. 685–689, 1996

0031-9422/96 $15.00 + 0.00

CHEMICAL MODIFICATION OF ESSENTIAL ARGININE RESIDUES ASSOCIATED WITH THE RED BEET (*BETA VULGARIS* L.) PLASMA MEMBRANE Ca^{2+}–ATPase

SWATI BASU and DONALD P. BRISKIN*

Department of Agronomy, University of Illinois, 1201 West Gregory Drive, Urbana, IL 61801, U.S.A.

(*Received in revised form* 23 *August* 1995)

Key Word Index—Transport ATPase; ATPase reaction mechanism, signal transduction; red beet; *Beta Vulgaris*; Chenopodiaceae.

Abstract—Two α-dicarbonyl reagents, phenylglyoxal and 2,3-butanedione, were used to demonstrate the presence of essential arginine residues in the mechanism of the plasma membrane Ca^{2+}–ATPase of red beet (*Beta vulgaris* L.) storage tissue. Both the ATP-dependent $^{45}Ca^{2+}$ transport and ITP hydrolytic activities of the Ca^{2+}–ATPase were inhibited by these reagents. Optimal inhibition was observed at pH 7.5 and 25°. Inhibition of ATP dependent $^{45}Ca^{2+}$ transport by phenylglyoxal and 2,3-butanedione was decreased by inclusion of ATP in the incubation medium. These results demonstrate that arginine residues are involved in the mechanism of the red beet plasma membrane Ca^{2+}–ATPase and may reside at the ATP binding region of the enzyme active site.

INTRODUCTION

The plasma membrane Ca^{2+}–ATPase is of considerable importance to the plant cell as it mediates extrusion of cytosolic calcium across the plasma membrane to the apoplast [1]. Together with Ca^{2+} transport systems associated with the endoplasmic reticulum and vacuole, this serves to maintain the low cytoplasmic Ca^{2+} concentration required for this divalent cation to function as a second messenger in signal transduction [1, 2]. At present, most studies on the plant plasma membrane Ca^{2+}–ATPase have focused on characterization of its enzyme activity and transport properties using isolated membrane vesicles [1, 3]. Due to its low abundance in the plasma membrane, this Ca^{2+}–ATPase has proved difficult to purify and little progress has been made in elucidating the structural details of this protein. Nevertheless, characterization of structure–function relationships for this protein will be essential for a complete understanding of its mechanism of ATP hydrolysis with linking to Ca^{2+} transport, and its regulation by factors such as calmodulin [1, 2 and refs cited therein].

One approach which could be utilized to identify amino acid moieties necessary for activity of the plant plasma membrane Ca^{2+}–ATPase involves the use of specific amino acid-modifying reagents [4]. In this respect, a number of studies using this approach have shown the presence of essential arginine groups associated with proteins which act on anionic substrates such as ATP [5–7 and refs cited therein]. These include several transport ATPases such as the mammalian Na^+, K^+–ATPase [8], the gastric H^+, K^+–ATPase [9], the plant plasma membrane H^+–ATPase [10] and the animal sarcoplasmic reticulum Ca^{2+}–ATPase [11]. It has been hypothesized that arginine has an important role in anion substrate binding since the guanidium group of this amino acid remains protonated and hence, positively charged within the pH range in which most enzymes remain stable and active [12]. Furthermore, this group is planar and could form multiple hydrogen bonds with phosphorylated substrates such as ATP [6, 13]. The α-dicarbonyl reagents, phenylglyoxal (PGO) and 2,3-butanedione (BD), have been successfully used to study the presence of arginine residues in the mechanism of a vast number of proteins [7 and refs cited therein]. According to Patthy and Theze [14], the specificity of these dicarbonyl reagents for essential arginine residues is explained by their lower pK_a at anion binding sites as compared to arginyl residues at other regions on the protein.

In this communication, we have examined the possible role of essential arginine residues in the mechanism of the plant plasma membrane Ca^{2+}–ATPase present in native plasma membrane vesicles. Our approach involved chemical treatment of the Ca^{2+}–ATPase using PGO and BD followed by the measurement of inhibitory effects upon $^{45}Ca^{2+}$ transport and enzyme activity. In addition, we evaluated the role of ATP and reaction ligands in modifying the inhibitory effects of these reagents.

*Author to whom correspondence should be addressed.

RESULTS AND DISCUSSION

The use of protein modification to elucidate essential amino acid moieties associated with the mechanism of an enzyme requires a two-step approach whereby a protein is derivatized by the modification reagent and then residual enzyme activity is determined under normal assay conditions [4]. Initial studies were conducted to establish conditions for optimal derivatization of the red beet plasma membrane Ca^{2+}–ATPase by the α-dicarbonyl reagents, PGO and BD. In our studies with the red beet Ca^{2+}–ATPase present in transport-competent plasma membrane vesicles, assays for activity would involve ATP-dependent $^{45}Ca^{2+}$ uptake as well as Ca^{2+}-dependent ITP hydrolysis. This latter assay is utilized because the plasma membrane Ca^{2+}–ATPase has a broad substrate specificity and can utilize GTP or ITP as substrates for activity [1, 2 and refs cited therein]. As the plant plasma membrane H^+–ATPase is substrate specific for ATP, this allows the assay for hydrolytic activity associated with the Ca^{2+}–ATPase even when the former enzyme is present in the same membrane preparation [15].

As shown in Fig. 1, when red beet plasma membrane fractions were treated with either 80 mM PGO or 300 mM BD, ATP-dependent $^{45}Ca^{2+}$ transport was inhibited, consistent with the involvement of essential arginine residues in the mechanism of this transport enzyme. However, the effectiveness of these two arginine modification reagents differed according to the pH of the incubation medium. While PGO appeared most effective when incubation with the inhibitor was conducted at low pH, the effectiveness of BD increased with an increase in the incubation pH. On the other hand, inhibition for both reagents relative to the activity recovered for the control sample appeared maximal at pH 7.5. This also represents the pH optimum for this enzyme [1 and refs cited therein]. The use of different buffering systems [BTP/Mes (*n*-morpholinoethanesulphonic acid), Tris/Mes or borate/Mes] during enzyme derivatization

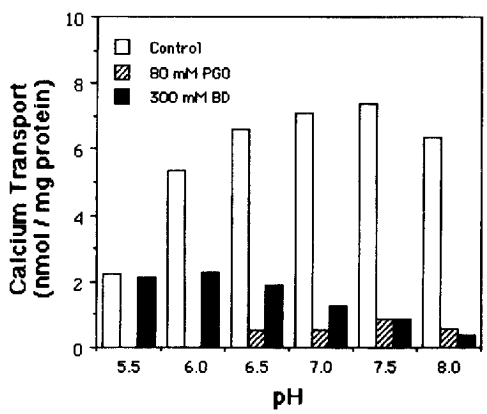

Fig. 1. Effect of incubation pH on the inhibition of the red beet plasma membrane Ca^{2+}–ATPase by BD or PGO. Treatment with either 300 mM BD or 80 mM PGO was conducted at 25° for 10 min in the presence of 25 mM borate/Mes buffer at the indicated pH. Aliquots were then taken for the assays of ATP-dependent $^{45}Ca^{2+}$ transport as described in Experimental.

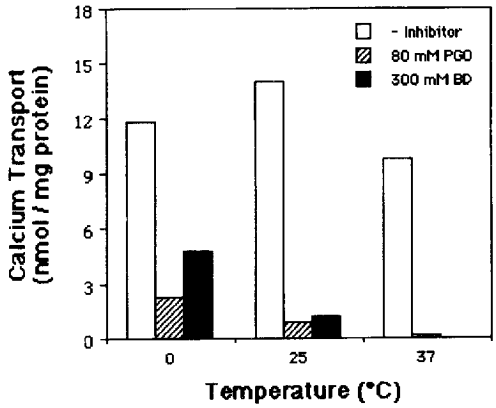

Fig. 2. Effect of temperature on the inhibition of the red beet plasma membrane Ca^{2+}–ATPase by BD or PGO. Incubation with either 80 mM PGO or 300 mM BD was conducted at 0°, 25° or 37° for 10 min in the presence of borate/Mes (pH 7.5). Following inhibitor treatment, aliquots were assayed for ATP-dependent $^{45}Ca^{2+}$ transport as described in Experimental.

showed only minor effects upon the degree of inhibition by both BD and PGO. Nevertheless, for both PGO and BD, inhibition was slightly enhanced by the presence of borate/Mes during derivatization. The effect of borate in promoting BD inhibition has been reported previously and is thought to occur because borate can stabilize the BD–arginine adduct [5, 6, 10, 16]. Maximal inhibition relative to the recovery of control $^{45}Ca^{2+}$ transport activity was also found to occur when incubation with either BD or PGO was conducted at 25° (Fig. 2). Based upon these results all subsequent modification reactions with BD and PGO were conducted in the presence of borate/MES buffer at pH 7.5 and 25°, prior to activity assays conducted under standard conditions (see Experimental).

The time course for inhibition of the red beet plasma membrane Ca^{2+}–ATPase by derivatization with various concentrations of BD or PGO exhibited a series of non-linear curves when log percentage residual $^{45}Ca^{2+}$ transport activity was plotted as a function of time (Fig. 3). Overall, PGO appeared to be more effective in inhibiting ATP-dependent $^{45}Ca^{2+}$ transport activity than BD. For BD concentrations exceeding 150 mM and PGO concentrations exceeding 20 mM, substantial inhibition occurred within the first 5 to 10 min of incubation with the reagent. Thereafter, the rate of inhibition declined substantially. From our previous studies with red beet plasma membrane fractions [10], it is apparent that these concentration levels of arginine modification reagents are well in excess of arginine groups available for derivatization. Therefore, the lack of a linear relationship on semi-logarithmic plots of residual activity versus time would suggest that more than one essential arginine group is likely being derivatized by these reagents. This is in contrast to what was previously observed for the plasma membrane H^+–ATPase from red beet [10] and mung bean [17] where treatment with BD or PGO resulted in a simple exponential decline of residual

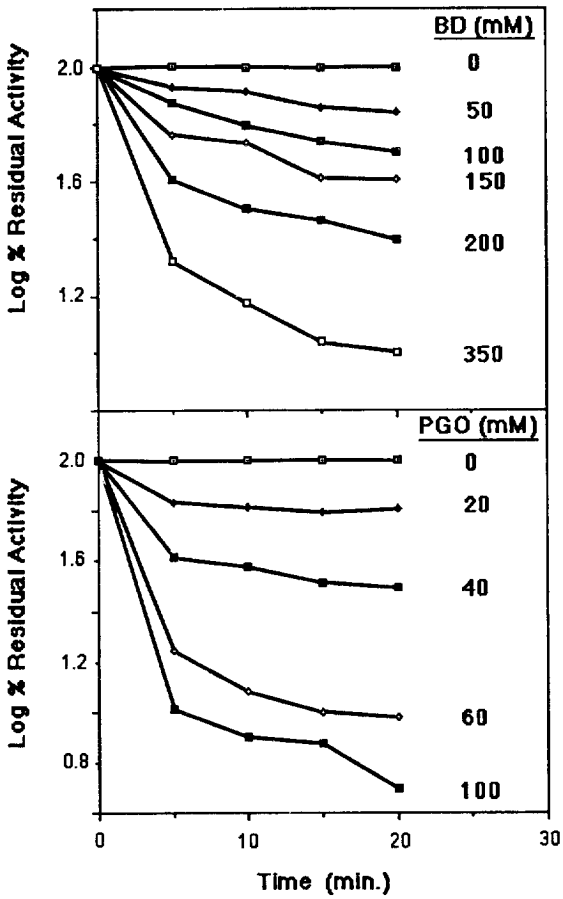

Fig. 3. Time course of BD and PGO inhibition of the red beet plasma membrane Ca^{2+}-ATPase. Treatment with the indicated concentration of BD or PGO was conducted according to Experimental. At the indicated times, aliquots were removed and assayed for ATP-dependent $^{45}Ca^{2+}$ transport.

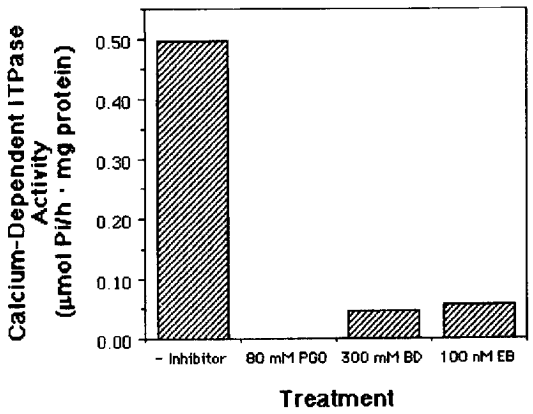

Fig. 4. Effect of BD or PGO on Ca^{2+}-dependent ITP hydrolysis activity associated with the red beet plasma membrane Ca^{2+}-ATPase. Plasma membrane fractions were treated with either 80 mM PGO or 300 mM BD for 10 min and then assayed for ITP hydrolysis as described in Experimental. For an underivatized control fraction, ITP hydrolysis was also measured in the presence of 100 nM erythrosin B (EB).

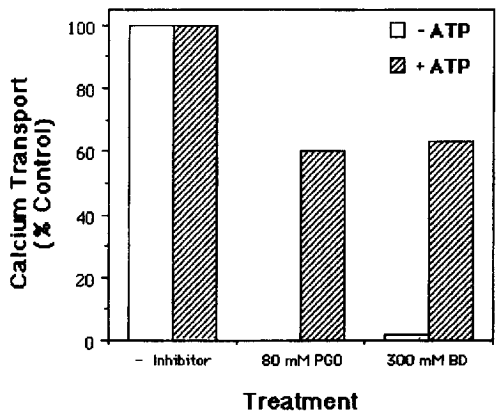

Fig. 5. Protection against BD or PGO inhibition of the red beet plasma membrane Ca^{2+}-ATPase by ATP. Plasma membrane fractions were treated with either 80 mM PGO or 300 mM BD in the presence or absence of 3.75 mM ATP. Following inhibitor treatment, ATP-dependent $^{45}Ca^{2+}$ transport was assayed as described in Experimental.

activity as a function of time. In these earlier studies this first-order decline of residual enzyme activity was interpreted to represent derivatization of a single type of arginine moiety associated with the active site of these enzymes.

Treatment with BD or PGO also inhibited the hydrolytic activity of the plasma membrane Ca^{2+}-ATPase (Fig. 4). As with ATP-dependent $^{45}Ca^{2+}$ transport activity, PGO was generally more effective than BD in arginine derivatization. That ITP hydrolysis was inhibited when 100 nM erythrosin B (EB) was included in a control assay further confirms that this enzyme activity is representative of the plasma membrane Ca^{2+}-ATPase. At this concentration level, EB has been shown to be a relatively specific inhibitor of the red beet plasma membrane Ca^{2+}-ATPase [1 and refs cited therein]. Because treatment with BD or PGO also inhibits the hydrolytic activity of the plasma membrane Ca^{2+}-ATPase, it is apparent that the inhibitory effects on ATP-dependent $^{45}Ca^{2+}$ transport do not result from an uncoupling of transport from ATP hydrolysis. Rather, inhibition results from a complete loss of enzyme function.

When incubation with BD or PGO was conducted in the presence of 3.75 mM ATP, substantial protection against inhibition by these reagents was observed (Fig. 5). For treatment with either 300 mM BD or 80 mM PGO, the level of protection by ATP was similar and amounted to about 60% of the control $^{45}Ca^{2+}$ transport activity. This is in contrast to the nearly complete inhibition of activity observed for PGO and BD treatment at these concentrations in the absence of ATP. That the enzyme substrate (ATP) can provide a substantial amount of protection against derivatization by BD or PGO would suggest that arginine residues derivatized by these reagents may reside at the active site of the plasma membrane Ca^{2+}-ATPase [4,7 and refs cited therein]. Alternatively, this result could be explained by the binding

of ATP at the active site, resulting in conformational changes which make arginine residues less susceptible to attack by BD or PGO.

Although the inclusion of 15 μM Ca^{2+} had relatively little effect upon PGO or BD treatment conducted in the presence of ATP, the presence of 3.75 mM Mg^{2+} resulted in about a 30% increase in inhibition by these reagents. This effect of Mg^{2+} in decreasing ATP protection against BD or PGO inhibition may result from conformational changes making essential arginines outside the ATP binding site more susceptable to BD or PGO derivatization.

CONCLUDING REMARKS

The results of this study demonstrate the presence of essential arginine residues in the mechanism of the red beet plasma membrane Ca^{2+}–ATPase. That the time course of enzyme inactivation at various concentrations of reagent displayed a non-linear relationship, when plotted in a semi-logarithimic manner, suggested that arginine residues with differing reactivity were modified by BD and PGO. For modification with either of these reagents, the ability of ATP to protect against inhibition would suggest that at least one essential arginine is associated with the active site of ATP hydrolysis.

An active site arginine residue reactive to BD and PGO has been previously demonstrated for a number of P-type transport ATPases including the mammalian Na^+, K^+–ATPase [8], the gastric H^+, K^+–ATPase [9], the plant plasma membrane H^+–ATPase [10, 17], the fungal plasma membrane H^+–ATPase [18] and the animal cell sarcoplasmic reticulum Ca^{2+}–ATPase [11]. In each case, protection against reaction of this residue with protein modification reagents has been observed in the presence of the ATP substrate and these authors have suggested that the substrate protectable arginine residue(s) derivatized by these reagents may be situated near the aspartic acid residue phosphorylated during the catalytic cycle of the enzyme. It should be noted that an additional role for arginine residues outside the active site region in Ca^{2+}–ATPases involves the interaction with acidic phospholipids, leading to an increase in affinity of the enzyme for Ca^{2+} and a stimulation of activity [19]. As the plant plasma membrane Ca^{2+}–ATPase is also stimulated by acidic phospholipids [20, 21], arginine residues could have a role in this function. Clearly, studies involving modification of purified plant plasma membrane Ca^{2+}–ATPase with radiolabelled PGO or BD, followed by radiolabelled peptide purification and sequence determination, could prove useful in elucidating the site(s) of modification by these reagents.

EXPERIMENTAL

Plant material and isolation of membrane vesicles. Red beets (*Beta vulgaris* L., cv. Detroit Dark Red) were purchased commercially. The beets were de-topped and stored at 4° in the dark for at least 10 days to ensure uniformity in membrane isolation [22]. Sealed plasma membrane vesicles were produced in large batch isolations from *ca* 500 g red beet storage tissue as described in ref. [10]. The plasma membrane-enriched fr. collected from the 26%/38% sucrose gradient interface was concd by centrifugation at 80 000 *g* for 35 min in a Beckman type 35 rotor. The pellet was suspended at a protein concn of *ca* 3 mg ml^{-1} in 250 mM sucrose, 1 mM Tris/Mes (pH 7.2), 1 mM DTE and either used immediately or frozen in liquid N_2 and stored at − 80°. Membrane vesicles stored in this manner retain both transport and ATPase activity for up to 3 months [23]. The level of ATP-dependent $^{45}Ca^{2+}$ uptake associated with batch membrane isolations varied from *ca* 7.1 to 15.8 nmol mg^{-1} protein 10 min^{-1}. This variability was likely due to the combined effects of differences in Ca^{2+}–ATPase mediated uptake and Ca^{2+} leakage from the vesicles.

Chemical modification of the plasma membrane Ca^{2+}-ATPase. Modification of arginine residues was conducted using PGO and BD. For Ca^{2+}-transport assays, modification with PGO or BD was carried out using 20 μg plasma membrane protein, incubated for 10 min at 25° in a 100 μl reaction vol. containing 250 mM borate/Mes (pH 7.5) and various concns of PGO or BD. This was followed by addition of 400 μl transport buffer containing 250 mM sorbitol, 100 mM KNO_3, 3.75 mM $MgSO_4$, 3.75 mM ATP, 0.4 mM NaN_3 and 18.75 μM $CaCl_2$ (2.5 mCi $^{45}Ca^{2+}$) and 2 mM L-arginine to prevent carry-over of reagent from incubation medium. For ITPase assays, incubation was similar to above. PGO was prepd in H_2O and boiled immediately prior to use.

Measurement of $^{45}Ca^{2+}$ transport. Ca uptake was measured by a modification of the method of ref. [24], using the uptake medium described in the previous section with the final Ca^{2+} concn being 15 μM (*ca* 10 μM free Ca^{2+}). Following 10 min incubation in the radiolabelled Ca^{2+} uptake assay, 150 μl aliquots were removed and the plasma membrane vesicles were collected by vacuum filtration on to 0.45 μm metricel membrane filters (Gelman). The filters were washed rapidly with 2 × 4 ml aliquots wash buffer (transport buffer without ATP and with cold 15 mM $CaCl_2$). Filtration was carried out using a manifold system consisting of 10 individual sintered glass filter support units (Microfiltration), individually valved to a common vacuum line. The filters were incubated in wash buffer for at least 20 min *in vacuo* prior to use. Radioactivity associated with the filters was determined by liquid scintillation spectroscopy in 5 ml scintillation cocktail (Bio-Safe).

Measurement of Ca^{2+}-dependent ITPase activity. Ca-dependent ITPase activity was determined by a modification of the method of ref. [15]. Plasma membrane vesicles (40 μg membrane protein ml^{-1}) were incubated for 20 min at 37°, in 200 mM sorbitol, 5 mM borate/Mes (pH 7.5), 80 mM KNO_3, 3 mM $MgSO_4$, 0.08 mM ammonium molybdate, 3 mM ITP (BTP salt, pH 7.5) and 1.6 mM L-arginine. Ca was supplied as 15 μM $CaCl_2$ (*ca* 10 μM free Ca^{2+}) in the absence or presence of 2 mM

EGTA (pH 7.5). The Pi released from ITP hydrolysis was determined colorimetrically according to the method of ref. [25]. Ca-dependent ITPase activity was evaluated as the difference between the activity measured in the presence and absence of Ca (EGTA present).

Protein assay. Protein was determined by the method of ref. [26], using bovine serum albumin as protein standard. The assay reagent was filtered just prior to use.

Any variations on these standard assay conditions are indicated in the figure legends. All experiments were repeated (× 3) and the data shown are for representative experiments where each measurement was conducted in triplicate. For each experimental measurement, variation did not exceed ± 5% of the mean.

Acknowledgement—Supported by DOE research grant no. DERG02-91ER20057 awarded to D.P.B.

REFERENCES

1. Briskin, D. P. (1990) *Plant Physiol.* **94**, 397.
2. Evans, D. E., Briars, S.-A. and Williams, L. E. (1991) *J. Exp. Botany* **42**, 285.
3. Briskin, D. P., Basu, S. and Ho, I. (1992) in *Transport and Receptor Proteins of Plant Membranes. Molecular Structure and Function* (Cooke, D. T. and Clarkson, D. T., eds), pp. 13–24. Plenum Press, New York.
4. Eyzaguirre, J. (1987) *Chemical Modification of Enzymes. Active Site Studies.* John Wiley, New York.
5. Riordan, J. F. (1973) *Biochemistry* **12**, 3915.
6. Riordan, J. F., McElvany, K. D. and Borders, C. L., Jr (1977) *Science* **195**, 884.
7. Lundblad, R. L. (1991) *Chemical Reagents for Protein Modification*, 2nd Edn. CRC Press, Boca Raton, FL.
8. Depont, J. J. H. H., Schoot, B. M., Van Prooyen-Van Eeden, A. and Bonting, S. L. (1977) *Biochim. Biophys. Acta* **482**, 213.
9. Schrijen, J. J., Luyben, W. and DePont, J. J. H. H. (1980) *Biochim. Biophys. Acta* **597**, 331.
10. Gildensoph, L. H. and Briskin, D. P. (1989) *Arch. Biochem. Biophys.* **271**, 254.
11. Murphy, A. J. (1976) *Biochem. Biophys. Res. Commun.* **70**, 1048.
12. Cohen, L. A. (1968) *Annu. Rev. Biochem.* **37**, 695.
13. Cotton, F. A., Hazen, E. E., Day, V. W., Larsen, S., Norman, J. G., Wong, S. T. K. and Johnson, K. H. (1973) *J. Am. Chem. Soc.* **95**, 2367.
14. Patthy, L. and Theze, J. (1970) *Eur. J. Biochem.* **105**, 387.
15. Carnelli, A., De Michelis, M. I. and Rasi-Caldogno, F. (1992) *Plant Physiol.* **98**, 1196.
16. Grisham, C. M. (1979) *Biochem. Biophys. Res. Commun.* **88**, 229.
17. Kasamo, K. (1988) *Plant Physiol.* **87**, 126.
18. Kasher, J. S., Allen, K. E., Kasamo, K. and Slayman, C. W. (1986) *J. Biol. Chem.* **261**, 10808.
19. Wuytack, F. and Raeymaekers, L. (1992) *J. Bioenerg. Biomembr.* **24**, 285.
20. Kasamo, K. and Nouchi, I. (1987) *Plant Physiol.* **83**, 323.
21. Hsieh, W.-L., Pierce, W. S. and Sze, H. (1991) *Plant Physiol.* **97**, 1535.
22. Poole, R. J., Briskin, D. P., Kratky, Z. and Johnstone, R. M. (1984) *Plant Physiol.* **74**, 549.
23. Giannini, J. L., Gildensoph, L. H. and Briskin, D. P. (1987) *Arch. Biochem. Biophys.* **254**, 621.
24. Giannini, J. L., Ruiz-Cristin, J. L. and Briskin, D. P. (1987) *Plant Physiol.* **85**, 1137.
25. Chifflet, S., Torriglia, A., Chiesa, R. and Tolosa, S. (1988) *Analyt. Biochem.* **168**, 1.
26. Bradford, M. M. (1979) *Analyt. Biochem.* **72**, 248.