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Identification of the Plasma Membrane Ca^{2+}-ATPase and of Its Autoinhibitory Domain[1,2]

Franca Rasi-Caldogno*, Antonella Carnelli, and Maria Ida De Michelis

Centro di Studio del Consiglio Nazionale delle Ricerche per la Biologia Cellulare e Molecolare delle Piante, Dipartimento di Biologia, Università di Milano, via G. Celoria, 26, 20133 Milano, Italy (F.R.-C., A.C.); and Istituto Botanico "Hanbury" ed Orto Botanico dell' Università Corso Dogali 1c, 16136 Genova, Italy (M.I.D.M.)

The effect of controlled proteolysis on the plasma membrane (PM) Ca^{2+}-ATPase was studied at the molecular level in PM purified from radish (*Raphanus sativus* L.) seedlings. Two new methods for labeling the PM Ca^{2+}-ATPase are described. The PM Ca^{2+}-ATPase can be selectively labeled by treatment with micromolar fluorescein isothiocyanate (FITC), a strong inhibitor of enzyme activity. Both inhibition of activity and FITC binding to the PM Ca^{2+}-ATPase are suppressed by millimolar MgITP. The PM Ca^{2+}-ATPase maintains the capability to bind calmodulin also after sodium dodecyl sulfate gel electrophoresis and blotting; therefore, it can be conveniently identified by ^{125}I-calmodulin overlay in the presence of calcium. With both methods a molecular mass of 133 kD can be calculated for the PM Ca^{2+}-ATPase. FITC-labeled PM Ca^{2+}-ATPase co-migrates with the phosphorylated intermediate of the enzyme—labeled by incubation with [γ-^{32}P]GTP in the presence of calcium—on acidic sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Controlled trypsin treatment of purified PM determines a reduction of the molecular mass of the PM Ca^{2+}-ATPase from 133 to 118 kD parallel to the increase of enzyme activity. Only the 133-kD but not the 118-kD PM Ca^{2+}-ATPase binds calmodulin. These results indicate that trypsin removes from the PM Ca^{2+}-ATPase an autoinhibitory domain that contains the calmodulin-binding domain of the enzyme.

Transient changes in the cytosolic Ca^{2+} concentration are involved in the response of plant cells to a number of hormonal and external signals. The role of Ca^{2+} as a second messenger is made possible not only by a fine regulation of opening and closing of Ca^{2+} channels but also, of course, by a fine regulation of active transport systems involved in its extrusion from the cytosol (Poovaiah and Reddy, 1993).

The PM Ca^{2+}-ATPase of a plant cell has all the characteristics to play a central role in the regulation of Ca^{2+} homeostasis of the cell and in particular to restore the very low cytosolic Ca^{2+} concentration of resting conditions after a signal-induced increase of its concentration (reviewed by Briskin, 1990; Evans et al., 1991; De Michelis et al., 1992; Evans, 1994). The PM Ca^{2+}-ATPase catalyzes a nH^+/Ca^{2+} antiport and so utilizes, besides the energy of hydrolysis of ATP, the proton electrochemical gradient built up by the PM H^+-ATPase. This allows Ca^{2+} extrusion from the cytoplasm to proceed far from equilibrium also against an electrochemical gradient that can be more than 60 kJ (Rasi-Caldogno et al., 1987; Miller et al., 1990). Moreover, kinetic characteristics of the enzyme are also important. In fact, it is half-saturated by Ca^{2+} in the micromolar range and has a very high affinity for MgATP (Rasi-Caldogno et al., 1989; Carnelli et al., 1992; De Michelis et al., 1992, 1993). Finally, the PM Ca^{2+}-ATPase activity is regulated by CaM, which strongly increases the V_{max} of the reaction, thus making the enzyme up-regulated by an increase of cytosolic Ca^{2+} and Ca^{2+}-CaM concentrations (Malatialy et al., 1988; Robinson et al., 1988; Williams et al., 1990; Erdei and Matsumoto, 1991; Carnelli et al., 1992; Rasi-Caldogno et al., 1992; De Michelis et al., 1993).

The last point has long been controversial. In fact, the PM Ca^{2+}-ATPase has a very high affinity for CaM; therefore, its stimulation by exogenous CaM is often very slight or even undetectable unless endogenous CaM is stripped by drastic treatments with Ca^{2+}-chelating agents (Williams et al., 1990; Rasi-Caldogno et al., 1993a). Moreover, a CaM-stimulated Ca^{2+}-ATPase is present also in endomembranes such as ER and tonoplast (Malatialy et al., 1988; Brauer et al., 1990; Hsieh et al., 1991; Askerlund and Evans, 1992; Chen et al., 1993; Gavin et al., 1993; Gilroy and Jones, 1993) and it has been proposed that the bulk of CaM-stimulated Ca^{2+}-ATPase is localized at endomembranes (Askerlund and Evans, 1992). The ratio between PM and endomembrane-associated CaM-stimulated Ca^{2+}-ATPase activities can vary in different plant tissues and can be difficult to establish correctly. In most cases only the transport activity of the enzymes has been determined, which is intrinsically misleading; in fact, ATP-dependent Ca^{2+} transport can be measured only in inside-out PM vesicles and the phase-partitioning technique used to purify plant PM produces mainly right-side-out PM vesicles. The PM and endomembrane-associated CaM-stimulated Ca^{2+}-ATPases have

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[2] Dedicated to Prof. Erasmo Marré on the occasion of his 75th birthday.

* Corresponding author; e-mail fimca@imiucca.csi.unimi.it; fax 39–2–26604399.

Abbreviations: Brij 58, polyoxyethylene-20-cetyl ether; BTP, bis-tris propane(1,3-bis[tris(hydroxymethyl)methylamino]-propane); CaM, calmodulin; FITC, fluorescein isothiocyanate; PM, plasma membrane.

fairly similar biochemical characteristics and their reported values of molecular mass fall in the same range (Evans et al., 1989; Williams et al., 1990; Hsieh et al., 1991; Askerlund and Evans, 1992, 1993; Chen et al., 1993; Thomson et al., 1993, 1994); therefore, they are not easily discerned.

The experimental system utilized in our laboratories, PM vesicles isolated from germinating radish (*Raphanus sativus* L.) seeds, has proven especially suitable to the study of the PM Ca^{2+}-ATPase. In radish seeds at the early stages of germination, the endomembrane system is poorly developed. In Suc-density gradients all of ATP-dependent Ca^{2+} transport and Ca^{2+}-dependent ATP-hydrolyzing activity co-migrate with PM markers and no activity associated with ER membranes is detectable (Rasi-Caldogno et al., 1987, 1989). In this material, tonoplast markers co-migrate with PM on Suc gradients but can be easily separated from PM by the aqueous two-phase partitioning technique (Pugliarello et al., 1991). An involvement of tonoplast-associated CaM-stimulated Ca^{2+}-ATPase in our system can be ruled out by the analysis of the distribution of tonoplast marker activities and CaM-stimulated Ca^{2+}-ATPase in membrane fractions purified by the aqueous two-phase partitioning technique (Carnelli et al., 1992; see also Table I). Finally, PM purified from germinating radish seeds are especially suitable to study not only the transport but also the hydrolytic activity of the PM Ca^{2+}-ATPase, exploiting the ability of the enzyme to utilize ITP as an alternative substrate, because Ca^{2+}-independent ITP hydrolysis is extremely low (Carnelli et al., 1992).

Using this experimental system, we have recently demonstrated that hydrolytic and transport activity of the PM Ca^{2+}-ATPase are stimulated by a controlled treatment with trypsin (Rasi-Caldogno et al., 1993a). Activation by proteolysis is both qualitatively and quantitatively similar to that determined by CaM. Moreover, the enzyme activated by CaM does not respond to activation by proteolysis, and the PM Ca^{2+}-ATPase activated by proteolysis is not further activated by CaM (Rasi-Caldogno et al., 1993a).

The aim of this work was to analyze the effect of controlled proteolysis on the PM Ca^{2+}-ATPase at the molecular level. Identification of the PM Ca^{2+}-ATPase on gel electrophoresis has been accomplished up to now by utilizing the characteristic of the enzyme to form a phosphorylated intermediate during the catalytic process (Williams et al., 1990; Askerlund and Evans, 1993; Thomson et al., 1993). In this work, to study the effect of controlled proteolysis on the PM Ca^{2+}-ATPase at the molecular level, besides following the migration of its phosphorylated intermediate in acidic gels, we have developed methods suitable also for SDS-PAGE according to Laemmli (1970), exploiting some characteristics of the enzyme. First, based on its high sensitivity to inhibition by fluorescein derivatives (Rasi-Caldogno et al., 1987, 1989; Graf and Weiler, 1989; Williams et al., 1990; Olbe and Sommarin, 1991; Carnelli et al., 1992; De Michelis et al., 1993), we have selectively labeled the PM Ca^{2+}-ATPase with FITC (Dux et al., 1986). Second, based on the high affinity of the PM Ca^{2+}-ATPase for CaM, we have labeled it with ^{125}I-CaM (James et al., 1989).

The reported results show that the molecular mass of the PM Ca^{2+}-ATPase is 133 kD and that the activation by controlled tryptic treatment is due to the cleavage of about 15 kD containing the CaM-binding domain of the enzyme.

MATERIALS AND METHODS

Preparation of PM Vesicles

Methods for 24-h-old radish (*Raphanus sativus* L. Tondo Rosso Quarantino, Ingegnoli, Milano, Italy) seed germination and microsome extraction were as described by Rasi-Caldogno et al. (1993b). PMs were purified by one-step partitioning in a 6.2% (w/w) dextran T500 (Pharmacia), PEG 3350 (Sigma) phase system containing 3 mM KCl and 5 mM potassium phosphate buffer, pH 7.8. The upper phase was incubated for 5 min on ice in the presence of 20 mM BTP-Hepes, pH 7.5, 3 mM ITP, 5 mM EDTA, and 0.1 mg/mL Brij 58 to strip endogenous CaM (Rasi-Caldogno et al., 1993a). The samples were diluted with 5 volumes of ice-cold medium containing 0.25 M Suc, 3 mM DTT, 5 mM EDTA, 0.1 mg/mL Brij 58, and 1 mM PMSF, pH 7, and the PM was collected by centrifugation at 48,000*g* for 35 min at 4°C. The pellets were resuspended in resuspension medium (0.25 M Suc, 0.5 mM DTT, 1 mM BTP-Hepes, pH 7) at about 4 to 5 mg of membrane proteins per mL, immediately frozen, and kept at −80°C until use. This procedure reproducibly yielded a purified PM fraction, similar to that previously described for the same plant material (De Michelis et al., 1991) but with a higher yield in PM. In fact, the U_1 fraction obtained had about 60% of the activities of the PM H^+-ATPase and of CaM-stimulated Ca^{2+}-ATPase and was 2- to 4-fold enriched in PM relative to endomembranes, as compared to the starting microsomes (Table I).

Table I. *Comparison of activities of markers of PM and endomembranes in microsomes and PM fraction (U_1) from radish seedlings*

Microsomes were partitioned in 6.2% dextran-PEG as described in "Materials and Methods." Microsomes and U_1 were treated with EDTA in the presence of Brij 58 as described in "Materials and Methods" to strip endogenous CaM, except for membranes used for assaying antimycin A-insensitive NADH-Cyt *c* reductase and oligomycin-sensitive ATPase activities, in which EDTA and Brij 58 were omitted. Enzyme assays were performed as described in "Materials and Methods"; activities are given in nmol min^{-1} g^{-1} fresh wt; values in parentheses represent specific activities (nmol min^{-1} mg^{-1} protein).

Enzyme	Microsomes	U_1
Protein (μg/g fresh wt)	110	56
Vanadate-sensitive ATPase[a]	45.3 (412)	28.1 (502)
Ca^{2+}-ATPase[b]		
Minus CaM	1.32 (12.0)	0.79 (14.1)
Plus CaM	3.13 (28.5)	1.94 (34.6)
K^+-dependent pyrophosphatase[a]	1.14 (10.4)	0.17 (3.0)
Oligomycin-sensitive ATPase[a]	3.80 (34.5)	0.76 (13.6)
Antymicin A-insensitive NAH-Cyt *c* reductase[b]	0.79 (7.2)	0.33 (5.9)

[a] Assays performed at 33°C. [b] Assays performed at 25°C.

Phosphorylated Intermediate Formation

PM vesicles were diluted about 10-fold with ice-cold washing medium (0.25 M Suc, 3 mM DTT, 0.1 mM EDTA, 1 mM BTP-Hepes, pH 7), centrifuged for 30 min at 48,000*g*, and resuspended in resuspension medium at about 4 mg of PM proteins per mL. The formation of ^{32}P-phosphorylated intermediate was assayed according to the method of Thomson et al. (1993) with some modifications. The reaction mixture (0.2 mL final volume) contained 12 μM $MgSO_4$, 50 mM KCl, 10 mM Hepes-KOH, pH 7, 50 μM $CaCl_2$ or 0.5 mM EGTA, and 0.1 μM [γ-^{32}P]GTP (250 μCi/nmol); 100 μM $LaCl_3$, 20 μg/mL CaM, and 1 μM eosin Y were included when indicated in the text. The reaction was initiated by adding 140 to 200 μg of PM proteins. After 15 s at 0°C the reaction was stopped by the addition of 0.4 mL of an ice-cold solution of 12% TCA, 1 mM GTP, and 50 mM NaH_2PO_4, incubated on ice for 15 min, and centrifuged for 5 min at 13,000*g*. Pellets were suspended in a cocktail of protease inhibitors (700 μg/mL tosyl-L-Lys chloromethyl ketone, 140 μg/mL leupeptin, 2.8 mM *p*-aminobenzamidine, 3.5 μg/mL soybean trypsin inhibitor) and centrifuged for 5 min at 13,000*g*. The effect of hydroxylamine was tested by suspending the TCA pellet in 100 mM Mes-KOH, pH 6, plus protease inhibitors in the presence or absence of 0.25 M hydroxylamine. After 30 min on ice the proteins were centrifuged for 5 min at 13,000*g*. All of the centrifugation steps were at 4°C. PMs were solubilized for acidic SDS-PAGE as described below.

Proteolytic Treatment

PM vesicles (1 mg of PM proteins per mL) were incubated for 5 min on ice in 0.1 mM EDTA, 0.5 mM ITP, 20 mM BTP-Hepes, pH 7, and 4 to 100 μg/mL trypsin. The reaction was blocked by addition of 2 mg/mL trypsin inhibitor. PMs were diluted about 10-fold with ice-cold washing medium supplemented with 0.5 mM ITP and 3 mM *p*-aminobenzamidine and collected by centrifugation at 48,000*g* for 30 min. Pellets were resuspended in resuspension medium at 4 mg of PM proteins per mL.

Treatment of the PM with FITC

FITC was freshly dissolved in *N,N*-dimethylformamide at 100-fold the final concentration used. PM proteins (4 mg/mL) were incubated in 50 μM $CaCl_2$, 20 mM BTP-Hepes, pH 7, in the presence of FITC at the concentrations specified in the figure legends. One percent *N,N*-dimethylformamide was present in all samples. After incubation for 15 min at 25°C, the samples were diluted with 10 volumes of ice-cold washing medium supplemented with 100 μg/mL Brij 58 and centrifuged for 30 min at 48,000*g*. The pellets were resuspended in resuspension medium at 4 mg of protein per mL.

SDS-PAGE

For acidic SDS-PAGE, PMs were solubilized for 1 h at 25°C with 10% glycerol, 1% SDS, 0.001% pyronine Y in 100 mM Na-phosphate buffer, pH 5.5. Ten millimolar DTT, 0.5 mM PMSF, and 5 μM chymostatin were freshly added to the solubilization buffer. Solubilized PM proteins (100 μg per lane) were subjected to slab-gel electrophoresis on a 5.6% polyacrylamide gel (2.7% cross-linker) containing 100 mM Na-phosphate buffer, pH 5.5, 0.1% SDS, 0.1% *N,N,N',N'*-tetramethylethylene, 0.1% ammonium persulfate (gel dimensions 13.5 × 14 × 0.15 cm, no stacking gel). The running buffer contained 100 mM Na-phosphate, pH 5.5, and 0.1% SDS. Electrophoresis was carried out at 170 mA constant current for about 3 h at 15°C. Following electrophoresis, gels were fixed for 5 min in 25% 2-propanol, 10% acetic acid, and 2% glycerol and dried overnight in a gel drier (Hoefer, San Francisco, CA) or blotted as described below. For ^{32}P autoradiography, the dried gels were exposed to Kodak X-Omat AR 5 autoradiography film using intensifying screens at −80°C for 3 to 7 d.

For standard SDS-PAGE according to Laemmli (1970), PMs were incubated for 5 min on ice in the cocktail of protease inhibitors and then solubilized for 60 min at 25°C in 4% SDS, 3% β-mercaptoethanol, 20% glycerol, 1 mM EDTA, 20 mM H_3PO_4 adjusted to pH 2.4 with Tris (2 mg of PM protein per mL). PM proteins (40 μg per lane) were subjected to slab-gel electrophoresis on a 7% polyacrylamide minigel (2.7% cross-linker) according to the method of Laemmli (1970).

Western Blot Analysis

After SDS-PAGE, the gel was blotted to an Immobilon NC/HA transfer membrane (Millipore) in 50 mM Tris, 38 mM Gly, 0.1% SDS, 20% methanol at 4°C. The membrane was blocked in 5% defatted milk powder, 0.2% Tween, 0.15 M NaCl, and 20 mM Tris-HCl, pH 7.4, washed, and incubated with anti-fluorescein rabbit IgG (H+L) fraction (catalog No. A-889; Molecular Probes, Sunnyvale, CA). Immunodetection was performed with a second antibody coupled to alkaline phosphatase (catalog No. A9919, Sigma).

^{125}I-CaM Overlay

^{125}I-CaM overlay was performed essentially as described by James et al. (1989). Briefly, after blotting the Immobilon membrane was incubated at 4°C overnight in blocking solution (5% defatted milk powder, 0.15 M NaCl in 20 mM Tris-HCl, pH 7.4). After washing, the membrane was incubated for 3 h at room temperature with 0.3 nM ^{125}I-CaM (100 μCi/μg), 3% BSA, 0.15 M NaCl, 20 mM Tris-HCl, pH 7.4, in the presence of 0.1 mM $CaCl_2$ or 2 mM EGTA and extensively washed in the respective buffers without CaM. Autoradiography of the dried membrane was performed with Kodak X-Omat AR 5 films using intensifying screens at −80°C for 24 to 72 h.

Assays of PM Ca^{2+}-ATPase Activity

The hydrolytic activity of the PM Ca^{2+}-ATPase was measured as Ca^{2+}-dependent MgITP hydrolysis (Carnelli et al., 1992). The standard assay medium contained 40 mM BTP-Hepes, pH 7, 50 mM KCl, 3 mM $MgSO_4$, 0.1 mM ammonium molybdate, 75 μg/mL Brij 58, 1 mM ITP, 1 mM EGTA plus

or minus $CaCl_2$ to give a free Ca^{2+} concentration of 30 μM (De Michelis et al., 1993). CaM was supplied at 20 μg/mL. Incubation was performed at 25°C for 1 h with about 50 μg of protein per mL.

Other Assays

Vanadate-sensitive ATPase and K^+-dependent pyrophosphatase were assayed as described by De Michelis et al. (1991) but Triton X-100 was substituted with Brij 58 (100 μg/mL). Antimycin A-insensitive NADH-Cyt *c* reductase activity was assayed as described by Rasi-Caldogno et al. (1987) but in the presence of 1 μM antimycin A. Membrane protein was assayed according to the method of Markwell et al. (1978).

RESULTS

Identification of the PM Ca^{2+}-ATPase on SDS-PAGE

In a first set of experiments we have exploited the ability of the PM Ca^{2+}-ATPase to form a phosphorylated intermediate to identify the enzyme on acidic SDS gels. Since the PM Ca^{2+}-ATPase also utilizes other nucleoside triphosphates instead of MgATP, [γ-^{32}P]GTP can be supplied to selectively label the PM Ca^{2+}-ATPase with respect to the H^+-ATPase, as already reported by Williams et al. (1990). Phosphorylation of PM proteins was performed at 0°C for 15 s in the presence of 0.1 μM [γ-^{32}P]GTP and the effects of Ca^{2+}, lanthanum, and eosin Y on phosphorylated intermediate formation were analyzed. As shown in Figure 1, in the absence of Ca^{2+} only a minor band of about 70 kD and a very faint one of about 88 kD are evident (lane 1), the

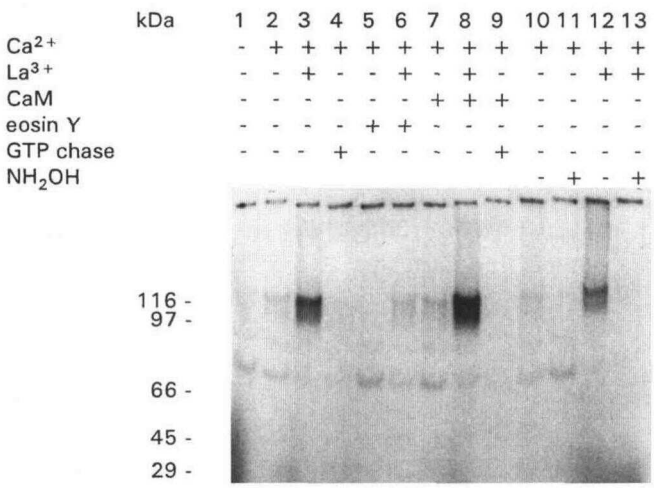

Figure 1. Characteristics of the phosphorylated intermediate of the PM Ca^{2+}-ATPase. Phosphorylation with [γ-^{32}P]GTP, PM washing, solubilization, acidic SDS-PAGE, and autoradiography were carried out as described in "Materials and Methods." Lane 1, 0.5 mM EGTA instead of 50 μM $CaCl_2$. When present, $LaCl_3$ was 100 μM, eosin Y was 1 μM, and CaM was 20 μg/mL. GTP chase, 1 min with 0.2 mM cold GTP after phosphorylation with [γ-^{32}P]GTP. Hydroxylamine treatment, TCA pellets were incubated in 100 mM Mes-KOH, pH 6, plus or minus 0.25 M hydroxylamine as described in "Materials and Methods."

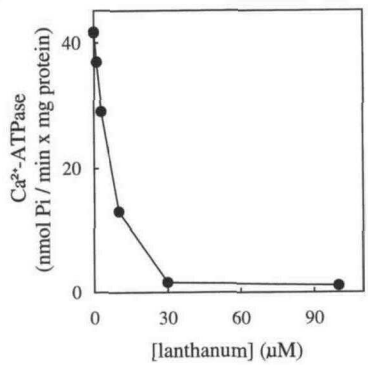

Figure 2. Inhibition of the PM Ca^{2+}-ATPase activity as a function of lanthanum concentration. PM Ca^{2+}-ATPase was measured in the presence of CaM as described in "Materials and Methods," except that calcium was supplied as 50 μM $CaCl_2$ in the absence of EGTA. Lanthanum was supplied as $LaCl_3$. Activity measured in the presence of 1 mM EGTA (8 nmol Pi min^{-1} mg^{-1} protein) was subtracted.

phosphorylation of which is unaffected by Ca^{2+}. In the presence of Ca^{2+}, two major ^{32}P-phosphoproteins of higher mol wt become evident (lane 2). Lanthanum, known to increase the steady-state level of phosphoenzyme of the PM Ca^{2+}-ATPase (Luterbacher and Schatzmann, 1983; Askerlund and Evans, 1993), greatly enhances the amount of the two Ca^{2+}-dependent ^{32}P-phosphoproteins (Fig. 1, lane 3) when supplied at a concentration that completely blocks the PM Ca^{2+}-ATPase activity (Fig. 2).

On the contrary, the fluorescein derivative eosin Y, which inhibits the PM Ca^{2+}-ATPase in a competitive manner with respect to nucleoside triphosphates (De Michelis et al., 1993), inhibits the formation of the two Ca^{2+}-dependent ^{32}P-phosphoproteins, both in the presence (Fig. 1, lane 6) and in the absence (Fig. 1, lane 5) of lanthanum. Moreover, when phosphorylation is performed in the presence of CaM, the amount of ^{32}P-phosphoproteins is increased both in the presence (Fig. 1, lane 8) and in the absence of (Fig. 1, lane 7) lanthanum. The two Ca^{2+}-dependent and CaM-stimulated phosphoproteins have a very high turnover. In fact labeling with [γ-^{32}P]GTP is completely washed out by a 1-min chase with cold GTP (Fig. 1, lanes 4 and 9). Moreover, the denaturated ^{32}P-phosphoproteins are sensitive to hydroxylamine (cf. lanes 10 and 11 in Fig. 1 for phosphorylation in the absence of lanthanum and lanes 12 and 13 for phosphorylation in the presence of lanthanum), indicating that the phosphorylation is on a carboxyl group such as that of an aspartic residue, characteristic of P-type ATPases (Schatzmann, 1989).

Taken together, these results indicate that the two Ca^{2+}-dependent, lanthanum-enhanced, eosin Y-inhibited, CaM-stimulated ^{32}P-phosphoproteins represent the phosphorylated intermediate of the PM Ca^{2+}-ATPase. The ratio between the two bands is rather variable in different membrane preparations, although the degree of purification of PM is high and reproducible; this suggests that the phosphoprotein of lower molecular mass could be a proteolytic product of the heavier one. Molecular masses of the two phosphoproteins estimated from different acidic gels ranged from 109 to 124 kD for that of higher molecular

mass and from 94 to 108 kD for that of lower molecular mass. The variability of molecular mass estimated in different experiments but not in adjacent lanes of the same gel, probably depending on scarce solubility of SDS at acidic pHs and incorrect negative charging of proteins, does not allow a clear-cut estimation of the mol wt of the PM Ca^{2+}-ATPase and is likely to be a source of variability of mol wt values reported in the literature for this enzyme (Williams et al., 1990; Askerlund and Evans, 1993; Thomson et al., 1993).

So, we decided to exploit the characteristic of the PM Ca^{2+}-ATPase as being specifically inhibited by low concentrations of fluorescein derivatives (Rasi-Caldogno et al., 1987, 1989; Graf and Weiler, 1989; Williams et al., 1990; Olbe and Sommarin, 1991; Carnelli et al., 1992; De Michelis et al., 1993) and to use FITC to specifically label the PM Ca^{2+}-ATPase. Because this modified dye binds to Lys residues of the protein in an alkali-stable covalent manner, it could serve to identify the PM Ca^{2+}-ATPase also on SDS gels run under standard (Laemmli, 1970) conditions.

To establish optimal conditions of FITC concentration and incubation duration for selectively labeling the PM Ca^{2+}-ATPase, we analyzed the kinetics of FITC inhibition of the PM Ca^{2+}-ATPase and of the PM H^+-ATPase. PMs were treated for 15 min at 25°C with increasing concentrations of FITC; excess FITC was washed out by centrifugation and the PM Ca^{2+}-ATPase or H^+-ATPase activities were measured. Figure 3 shows that in our conditions treatment with 0.3 to 300 μM FITC severely inhibited the Ca^{2+}-ATPase: 50% inhibition of the PM Ca^{2+}-ATPase was observed after treatment with about 2 μM FITC. No inhibition of the PM H^+-ATPase was observed in the presence of up to 300 μM FITC. Thus, FITC in the micromolar concentration range can be used to selectively label the PM Ca^{2+}-ATPase, even if the H^+-ATPase is at least 10 times more abundant in the PM.

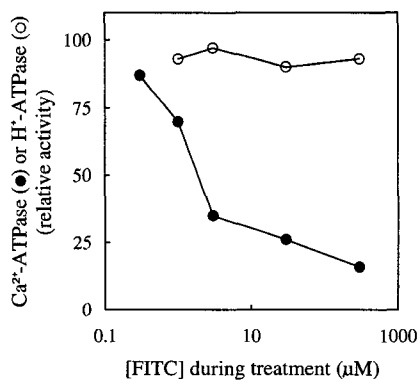

Figure 3. Effect of treatment with increasing concentrations of FITC on PM Ca^{2+}-ATPase (●) and on PM H^+-ATPase (○) activities. Treatment with increasing FITC concentrations for 15 min at 25°C and PM washing were performed as described in "Materials and Methods." PM Ca^{2+}-ATPase was assayed in the presence of CaM as described in "Materials and Methods"; H^+-ATPase was assayed at pH 7.0 and 25°C as previously described (De Michelis et al., 1993). Control activities were 34 and 210 nmol Pi min^{-1} mg^{-1} protein, respectively, for PM Ca^{2+}-ATPase and PM H^+-ATPase.

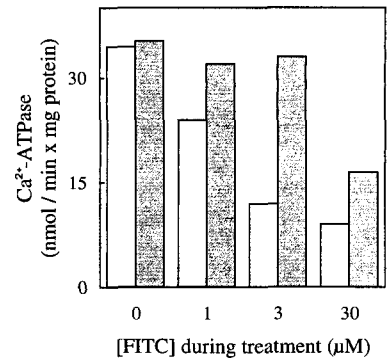

Figure 4. Protection by MgITP against inhibition of PM Ca^{2+}-ATPase by FITC. Experimental conditions were as in Figure 3, but FITC treatment was performed in the absence (open bars) or in the presence (shaded bars) of 3 mM MgITP.

Fluorescein derivatives inhibit the PM Ca^{2+}-ATPase in a competitive manner with respect to Mg-triphosphopurine nucleosides (De Michelis et al., 1993). Figure 4 shows that the effect of FITC was inhibited by the presence of MgITP during FITC treatment in a concentration-dependent manner. Inhibition by FITC up to 3 μM was abolished by the presence of 3 mM MgITP; at higher concentrations of FITC, 3 mM MgITP only partially abolished FITC inhibition.

FITC-labeled proteins can be immunodetected, after separation of PM proteins on SDS-PAGE and western blotting, with an antibody against FITC. Figure 5 shows that in solubilized PM treated with 2 μM FITC, the anti-FITC antibody recognized, besides other minor bands, two major proteins of 133 and 118 kD (lanes 1 and 3). The intensity of these bands was markedly lower when PM treatment with FITC was performed in the presence of MgITP (lanes 2 and 4). So, conditions in which MgITP effectively competes with FITC and protects the PM Ca^{2+}-ATPase from FITC inhibition (Fig. 4) are effective also in inhibiting the binding of FITC to the 133- and 118-kD proteins (Fig. 5).

Eosin Y, another fluorescein derivative that selectively inhibits the PM Ca^{2+}-ATPase at low concentrations (De Michelis et al., 1993), was used to analyze its capability to compete with FITC for binding to the 133- and 118-kD proteins. Figure 6 shows that eosin Y effectively inhibited FITC binding to the two proteins: inhibition was clearly evident with as little as 0.1 μM eosin Y (lane 2) and increased with the increase of eosin Y concentrations (lanes 3–5).

Together, these data all suggest that the 133- and the 118-kD FITC-labeled proteins represent the PM Ca^{2+}-ATPase. Also, analogous to what has been observed for the phosphorylated intermediate of the PM Ca^{2+}-ATPase, the ratio between the intensity of the two bands is variable in different PM preparations. These observations support the view that the protein with lower mol wt may represent a product of hydrolysis of the heavier one.

Molecular masses of the FITC-labeled Ca^{2+}-ATPase proteins are much higher than those calculated for the phosphorylated intermediate of the PM Ca^{2+}-ATPase in acidic gels. To check whether the FITC-labeled proteins are indeed the same that form the phosphorylated intermediate,

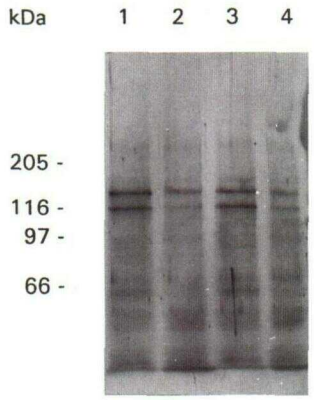

Figure 5. Labeling of the PM Ca^{2+}-ATPase by FITC and protection by MgITP. Treatment of PM with 2 μM FITC was performed as described in "Materials and Methods," in the absence (lanes 1 and 3) or in the presence (lanes 2 and 4) of 3 mM MgITP. Laemmli SDS-PAGE, blotting, and immunodetection were as described in "Materials and Methods."

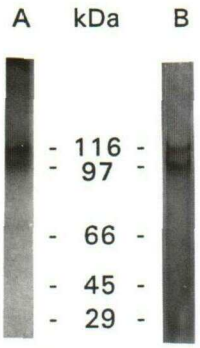

Figure 7. Co-migration of ^{32}P-labeled and FITC-labeled PM Ca^{2+}-ATPase in acidic SDS-PAGE. PMs were labeled as described in "Materials and Methods" with [γ-^{32}P]GTP in the presence of 50 μM $CaCl_2$ and 100 μM $LaCl_3$ (A) or with 2 μM FITC (B). After acidic SDS-PAGE, half of the gel was dried and autoradiographed for ^{32}P-labeled PM and half was blotted and immunodetected for FITC-labeled PM, as described in "Materials and Methods."

we labeled PM with FITC or with [γ-^{32}P]GTP and separated PM proteins on the same acidic gel. Figure 7 shows that the two FITC-labeled proteins migrated in acidic SDS-PAGE exactly as the two ^{32}P-phosphoproteins. This result confirms that the 133- and 118-kD FITC-labeled proteins are indeed the PM Ca^{2+}-ATPase and that molecular masses of proteins determined on acidic gels should be taken with caution.

Analysis of Molecular Changes Induced by Controlled Proteolysis

We have previously reported that controlled treatment with trypsin activates the PM Ca^{2+}-ATPase and makes it insensitive to further activation by CaM (Rasi-Caldogno et al., 1993a). To study the effect of trypsin treatment at the molecular level, PMs (1 mg of PM protein per mL) were treated with increasing concentrations of trypsin for 5 min at 0°C. After addition of an excess of trypsin inhibitor, PMs were pelleted and used for Ca^{2+}-ATPase assay or treated either with [γ-^{32}P]GTP for phosphorylated intermediate detection on acidic SDS-PAGE or with FITC for immunodetection after SDS-PAGE according to the method of Laemmli (1970) and blotting.

Figure 8 shows the effect of PM treatment with increasing concentrations of trypsin on the ^{32}P-phosphorylated intermediate, measured in both the presence (left) and absence (right) of lanthanum, and on the activity of the PM Ca^{2+}-ATPase. Increasing trypsin concentrations led to a progressive decrease of the ^{32}P-phosphoprotein at higher mol wt, paralleled by an increase of the ^{32}P-phosphoprotein at lower mol wt. The higher mol wt phosphoprotein was evident only with trypsin concentrations (4–8 μg/mg PM protein) that only partially activated the PM Ca^{2+}-ATPase (lanes 2, 3, and 8). The further increase of trypsin concentration to 16 μg/mg PM proteins rendered hardly distinguishable (lanes 4 and 9) the upper band, which completely disappeared at the highest concentrations of trypsin tested (32 and 100 μg/mg PM proteins, lanes 5, 6, and 10), which induced maximal activation of the PM Ca^{2+}-ATPase. So, kinetics of activation of PM Ca^{2+}-ATPase and kinetics of decrease of upper- and increase of lower-band intensities are absolutely comparable.

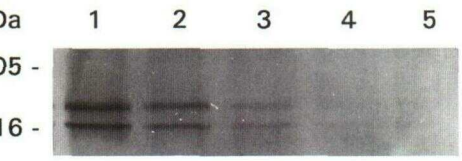

Figure 6. Inhibition of FITC labeling of the PM Ca^{2+}-ATPase by eosin Y. Treatment of PM with 2 μM FITC was performed as described in "Materials and Methods" in the absence (lane 1) or in the presence of 0.1 (lane 2), 0.5 (lane 3), 2 (lane 4), or 10 μM eosin Y (lane 5). Laemmli SDS-PAGE, blotting, and immunodetection were as described in "Materials and Methods."

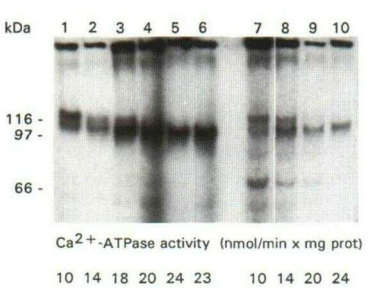

Figure 8. Effect of controlled proteolysis on activity of the PM Ca^{2+}-ATPase and on molecular mass of its phosphorylated intermediate. PMs (1 mg PM proteins [prot] per mL) were treated with increasing concentrations of trypsin and washed as described in "Materials and Methods": lanes 1 and 7, no trypsin; lanes 2 and 8, 4 μg/mL; lane 3, 8 μg/mL; lanes 4 and 9, 16 μg/mL; lanes 5 and 10, 32 μg/mL; lane 6, 100 μg/mL. Ca^{2+}-ATPase activity was assayed as described in "Materials and Methods" but in the absence of CaM. Ca^{2+}-dependent ^{32}P-phosphorylated intermediate formation was assayed as described in "Materials and Methods," in the presence of 50 μM $CaCl_2$ plus (lanes 1–6) or minus (lanes 7–10) 100 μM $LaCl_3$. Acidic SDS-PAGE and autoradiography were as described in "Materials and Methods."

The same picture is obtained when PM proteins are labeled with FITC after trypsin treatment. Figure 9 shows that also in this case treatment with 4 μg/mL trypsin induced a decrease of the upper band intensity and an increase of the lower one (lanes 3 and 6). Moreover, only the low mol wt band was evident when PMs were treated with a concentration of trypsin (32 μg/mL, lanes 1 and 4) that induced maximal activation of the PM Ca^{2+}-ATPase (Fig. 8).

These results taken together indicate that controlled tryptic treatment determines a reduction of the molecular mass of the PM Ca^{2+}-ATPase (estimated from SDS-PAGE according to the method of Laemmli [1970]) from 133 to 118 kD. The 15-kD fragment cleaved by trypsin contains an autoinhibitory domain of the enzyme.

Moreover, these results confirm that the 118-kD protein present in undigested PM represents a proteolytic product of the 133-kD protein. The presence of a significant and variable amount of 118-kD protein in undigested PM can explain the rather variable activation (between 150 and 300% stimulation) of the PM Ca^{2+}-ATPase induced by controlled trypsin pretreatment of different PM preparations (Rasi-Caldogno et al., 1993a).

Nature of the Autoinhibitory Domain of PM Ca^{2+}-ATPase

The activation of the PM Ca^{2+}-ATPase induced by controlled tryptic treatment of PM is qualitatively and quantitatively similar to that induced by CaM. Moreover, the trypsin-activated enzyme loses the sensitivity to CaM and the CaM-activated enzyme is not further activated by trypsin (Rasi-Caldogno et al., 1993a). This suggests that the PM Ca^{2+}-ATPase of higher plants, like the PM Ca^{2+}-ATPase of animal cells (Carafoli, 1991, and refs. therein), contains an autoinhibitory domain that binds CaM. Binding of CaM to this domain or its removal through controlled proteolysis led the PM Ca^{2+}-ATPase to the same activated state.

The CaM-binding domain of the PM Ca^{2+}-ATPase of animal cells maintains the capability to bind CaM in a Ca^{2+}-dependent manner also after separation of the enzyme on SDS-PAGE and western blotting (James et al., 1989; Carafoli, 1991). The same behavior has been observed for a CaM-stimulated Ca^{2+}-ATPase of unknown membrane localization partially purified from microsomes of maize coleoptiles (Evans et al., 1989). We tested this capability for the PM Ca^{2+}-ATPase from radish seedlings. PM proteins were separated on SDS-PAGE, blotted, and overlaid with ^{125}I-CaM plus or minus Ca^{2+}. Figure 10 shows that ^{125}I-CaM labeled only one PM protein and this labeling was absolutely dependent on the presence of Ca^{2+}. The molecular mass of the ^{125}I-CaM-labeled protein was 133 kD, which is equal to that of the FITC-labeled undigested PM Ca^{2+}-ATPase.

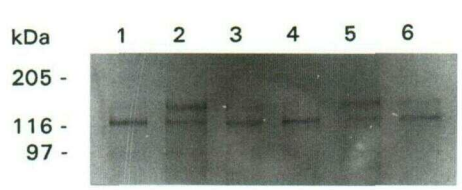

Figure 9. Decrease of molecular mass of FITC-labeled PM Ca^{2+}-ATPase induced by controlled proteolysis. PMs (1 mg PM proteins per mL) were treated with 0 (lanes 2 and 5), 4 (lanes 3 and 6), or 32 μg/mL trypsin (lanes 1 and 4), as described in "Materials and Methods." Treatment with 2 μM FITC, Laemmli SDS-PAGE, blotting, and immunodetection were as described in "Materials and Methods."

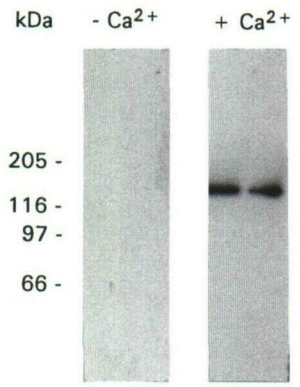

Figure 10. Ca^{2+}-dependent ^{125}I-CaM labeling of the PM Ca^{2+}-ATPase. Separation of PM proteins on Laemmli SDS-PAGE, blotting, ^{125}I-CaM overlay (in the presence of 2 mM EGTA or 100 μM $CaCl_2$), and autoradiography were performed as described in "Materials and Methods."

When an ^{125}I-CaM overlay was made on PM treated with trypsin in the same conditions as in Figure 8, the labeling with ^{125}I-CaM of the PM Ca^{2+}-ATPase decreased as a function of trypsin concentration (Fig. 11). Binding of ^{125}I-CaM was nearly abolished by the maximal trypsin concentration tested (32 μg/mg PM protein), i.e. by a concentration inducing maximal activation of the PM Ca^{2+}-ATPase (Fig. 8) and complete loss of sensitivity to CaM (Rasi-Caldogno et al., 1993a). These results indicate that the autoinhibitory polypeptide cleaved by trypsin treatment contains at least part of the CaM-binding domain of the plant cell PM Ca^{2+}-ATPase.

DISCUSSION

The results reported in this paper demonstrate that the PM Ca^{2+}-ATPase of plant cells can be selectively identified after separation of purified PM by SDS-PAGE with three different methods, based on different properties of the

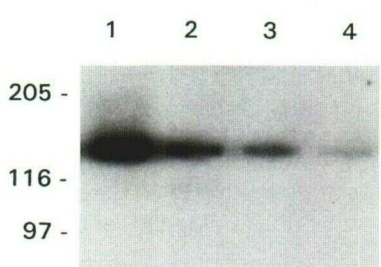

Figure 11. Decrease of ^{125}I-CaM labeling of the PM Ca^{2+}-ATPase induced by controlled proteolysis. PMs (1 mg PM proteins per mL) were treated with 0 (lane 1), 4 (lane 2), 8 (lane 3), or 32 μg/mL trypsin (lane 4) as described in "Materials and Methods." Laemmli SDS-PAGE, blotting, ^{125}I-CaM overlay, and autoradiography were as described in "Materials and Methods."

enzyme. As previously reported (Williams et al., 1990), formation of a phosphorylated intermediate during the catalytic cycle and low specificity for triphosphopurine nucleosides allow selective labeling of the PM Ca^{2+}-ATPase with [γ-^{32}P]GTP. We have exploited the high sensitivity of the enzyme to inhibition by fluorescein derivatives (Rasi-Caldogno et al., 1987, 1989; De Michelis et al., 1993) to selectively label the catalytic site with FITC (Dux et al., 1986). Finally, the PM Ca^{2+}-ATPase retains the ability to bind CaM after SDS-PAGE and thus can be labeled by ^{125}I-CaM overlay (Evans et al., 1989; James et al., 1989).

Both [γ-^{32}P]GTP and FITC label two distinct proteins, one about 15 kD smaller than the other, which both represent the PM Ca^{2+}-ATPase, as indicated by the characterization of phosphorylated intermediate formation and of FITC binding. ^{125}I-CaM labels only the heavier of the two proteins labeled by the other two probes. Identity of the proteins labeled by the three different methods is indicated by the fact that both of the bands labeled by [γ-^{32}P]GTP and by FITC co-migrate on acidic SDS-PAGE and that the heavier one co-migrates with that labeled by ^{125}I-CaM overlay on standard (Laemmli, 1970) SDS-PAGE. As discussed below, the lower molecular mass protein labeled by [γ-^{32}P]GTP and by FITC, but not by ^{125}I-CaM overlay, represents the product of proteolysis of the heavier one and is present also in undigested PM due to endogenous proteolysis.

The values of molecular mass of the PM Ca^{2+}-ATPase calculated from acidic SDS-PAGE are 109 to 124 and 94 to 108 kD, respectively, for the entire and proteolyzed protein. These values are in the same range as those previously determined under similar conditions for the PM Ca^{2+}-ATPase from other plant materials (Williams et al., 1990; Askerlund and Evans, 1993; Thomson et al., 1993). However, quite different values are obtained from standard (Laemmli, 1970) SDS-PAGE: 133 and 118 kD, respectively, for the entire and proteolyzed PM Ca^{2+}-ATPase. Discrepancy between molecular mass values determined in acidic or in standard SDS-PAGE and high variability of estimation in acidic SDS-PAGE have already been reported (Askerlund and Evans, 1993; Thomson et al., 1993) and likely depend on the low solubility of SDS at acidic pHs, which leads to incomplete negative charging of the proteins and thus to formation of broad bands and to nonlinear migration of mol wt marker proteins. The use of alternative methods for identification of the PM Ca^{2+}-ATPase like FITC labeling and ^{125}I-CaM overlay, which are compatible with standard SDS-PAGE, has allowed a much more reliable determination of the molecular mass of the PM Ca^{2+}-ATPase, which is 133 kD. This result, together with a functional mol wt of 270,000 as estimated by radiation inactivation (Rasi-Caldogno et al., 1990), indicates that the plant PM Ca^{2+}-ATPase operates as a dimer.

The analysis of phosphorylated intermediate formation presented in this paper establishes two, important characteristics of the PM Ca^{2+}-ATPase. The level of Ca^{2+}-dependent phosphorylated intermediate is greatly enhanced by lanthanum, an effect that likely reflects inhibition by lanthanum of phosphorylated intermediate cleavage (Luterbacher and Schatzmann, 1983). The strong effect of lanthanum and the very low level of Ca^{2+}-dependent phosphorylated intermediate in its absence suggests that the PM Ca^{2+}-ATPase of plants shares with the PM Ca^{2+}-ATPase of animal cells a ratio between rate of phosphorylation and hydrolysis of phosphorylated intermediate, which determines a steady-state level of phosphorylated intermediate lower than that of other P-type ATPase, such as the Ca^{2+}-ATPase of sarcoplasmic reticulum (Schatzmann, 1989; Carafoli, 1991, and refs. therein). Moreover, the fact that CaM increases the phosphorylated intermediate level both in the presence and in the absence of lanthanum suggests that CaM stimulates the rate of formation of phosphorylated intermediate rather than its breakdown.

Last but not least, another important aspect of this work is the analysis at the molecular level of the effect of proteolytic treatments with trypsin, which activate the PM Ca^{2+}-ATPase and make it unresponsive to CaM (Rasi-Caldogno et al., 1993a). Treatment of PM with increasing concentrations of trypsin, which progressively activate the PM Ca^{2+}-ATPase, determines the progressive disappearance of the 133-kD protein and its transformation into the 118-kD protein. Only the 133-kD protein but not the 118-kD one is capable of binding CaM. These results provide, to our knowledge, the first demonstration that trypsin treatment of the PM selectively cleaves an autoinhibitory domain of the plant PM Ca^{2+}-ATPase, which contains the CaM-binding site of the enzyme, or at least part of it.

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