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Purification of the Plasma Membrane Ca^{2+}-ATPase from Radish Seedlings by Calmodulin-Agarose Affinity Chromatography[1]

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The Ca^{2+}-ATPase of the plasma membrane (PM) of germinating radish (*Raphanus sativus* L.) seeds was purified by calmodulin (CaM)-affinity chromatography using a batch procedure. PM purified by aqueous two-phase partitioning was solubilized with *n*-dodecyl β-D-maltoside and applied to a CaM-agarose matrix. After various washings with decreasing Ca^{2+} concentrations, the Ca^{2+}-ATPase was eluted with 5 mM ethylenediaminetetraacetate (EDTA). The EDTA-eluted fraction contained about 25% of the loaded Ca^{2+}-ATPase activity, with a specific activity 70-fold higher than that of the starting PM fraction. The EDTA-eluted fraction was highly enriched in a 133-kD polypeptide, which was identified as the PM Ca^{2+}-ATPase by ^{125}I-CaM overlay and fluorescein-isothiocyanate labeling. The PM Ca^{2+}-ATPase cross-reacted with an antiserum against a putative Ca^{2+}-ATPase of the *Arabidopsis thaliana* chloroplast envelope.

Ca^{2+}-pumping ATPases play a crucial role in maintaining Ca^{2+} homeostasis and in restoring it after the increase of cytosolic Ca^{2+} concentration brought about by different stimuli. Given the limited capacity of internal stores, the PM Ca^{2+}-ATPase is particularly important in long-term control, especially to extrude Ca^{2+} entering the cell due to the opening of PM Ca^{2+} channels (Briskin, 1990; Evans et al., 1991; De Michelis et al., 1992; Poovaiah and Reddy, 1993; Ranjeva et al., 1993; Askerlund and Sommarin, 1996).

The PM Ca^{2+}-ATPase is a P-type ATPase that catalyzes an H^+/Ca^{2+} exchange (Rasi-Caldogno et al., 1987; Briskin, 1990; Evans et al., 1991; De Michelis et al., 1992; Askerlund and Sommarin, 1996). The PM Ca^{2+}-ATPase activity is stimulated by CaM, which binds to an autoinhibitory domain of the enzyme, causing a strong increase in the V_{max} and a decrease in the apparent K_m for free Ca^{2+} (Malatialy et al., 1988; Robinson et al., 1988; Williams et al., 1990; Erdei and Matsumoto, 1991; Rasi-Caldogno et al., 1992, 1993, 1995; De Michelis et al., 1993; Kurosaki and Kaboraki, 1994). All of these characteristics resemble those of the PM Ca^{2+}-ATPase of mammals (Carafoli, 1991). However, in contrast to mammals, plant PM-type Ca^{2+}-ATPases are also present in endomembranes, and are often more abundant in endomembranes than in the PM (for review, see Evans, 1994; Askerlund and Sommarin, 1996). Moreover, the Ca^{2+}-ATPase of the PM of plant cells has a very high affinity for CaM, so that stimulation by exogenous CaM is low or undetectable unless endogenous CaM is stripped by drastic treatments with Ca^{2+}-chelating agents (Williams et al., 1990; Evans et al., 1992; Rasi-Caldogno et al., 1993).

CaM-stimulated Ca^{2+}-ATPases of endomembranes and of PM have similar biochemical characteristics, and cannot be easily discerned (Hsieh et al., 1991; Askerlund and Evans, 1993; Thomson et al., 1993, 1994; Askerlund, 1996; Askerlund and Sommarin, 1996; Hwang et al., 1997). The main reported differences between the two types of plant CaM-stimulated Ca^{2+}-ATPases are: (a) the sensitivity of the endomembrane enzymes to inhibition by fluorescein derivatives is slightly lower than that of the PM enzyme (Thomson et al., 1993; Bush and Wang, 1995; Askerlund and Sommarin, 1996), and (b) the molecular weight of the PM enzyme is higher than that of endomembrane CaM-stimulated Ca^{2+}-ATPases (Thomson et al., 1993; Askerlund, 1996; Askerlund and Sommarin, 1996; Hwang et al., 1997).

Since the first report by Dieter and Marmè (1981), CaM-stimulated Ca^{2+}-ATPases have been purified from different plant materials by CaM-affinity chromatography, but all of them were localized on endomembranes (Briars et al., 1988; Evans et al., 1989, 1992; Askerlund and Evans, 1992; Theodoulou et al., 1994; Askerlund, 1996; Hwang et al., 1997).

Germinating radish seeds are an experimental system particularly suitable for studying the PM-localized Ca^{2+}-ATPase (Rasi-Caldogno et al., 1995). In fact, the endomembrane system is poorly developed, and all of the Ca^{2+}-ATPase activity is localized on the PM (Rasi-Caldogno et al., 1987, 1989). Furthermore, highly purified PM vesicles are easily obtained by the aqueous two-phase partitioning

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Abbreviations: Brij 58, polyoxyethylene-20-cetyl ether; CaM, calmodulin; FITC, fluorescein isothiocyanate; PM, plasma membrane.

technique, with quite a high yield (De Michelis et al., 1991; Rasi-Caldogno et al., 1995). Ca^{2+}-ATPase activity in the PM is among the highest reported and can be easily monitored both as nucleoside-triphosphate-dependent Ca^{2+} transport and as Ca^{2+}-dependent ITPase activity (Carnelli et al., 1992).

Here we show that affinity chromatography on CaM-agarose with a batch procedure purifies the PM Ca^{2+}-ATPase of radish seeds by 70-fold, with a recovery of about 25%.

MATERIALS AND METHODS

Preparation of PM Vesicles

Methods for radish (*Raphanus sativus* L. Tondo Rosso Quarantino, Ingegnoli, Milano, Italy) seed germination, microsome extraction, and PM purification were as described previously (Rasi-Caldogno et al., 1995). CaM stripping was performed by incubating the upper phase for 10 min on ice in the presence of 20 mM BTP (1,3-bis[Tris (hydroxymethyl)methylamino]-propane)-Hepes, pH 7.5, 3 mM ITP, 30 mM EDTA, and 0.1 mg mL^{-1} Brij 58. The samples were diluted with 5 volumes of ice-cold medium containing 0.25 M Suc, 3 mM DTT, 0.1 mg mL^{-1} Brij 58, 1 mM PMSF, and 1 mM BTP-Hepes, pH 7.0, and the PM was collected by centrifugation at 48,000*g* for 35 min at 4°C. The pellets were placed in resuspension medium (10% [v/v] glycerol, 0.5 mM DTT, and 1 mM Mops-KOH, pH 7.0) at 6 to 8 mg of membrane proteins per mL, immediately frozen, and kept at −80°C until use.

Solubilization of PM Ca^{2+}-ATPase

To solubilize PM Ca^{2+}-ATPase, PM vesicles were incubated with *n*-dodecyl β-D-maltoside (4 mg detergent mL^{-1}: 4 mg protein mL^{-1}, unless otherwise specified) for 15 min on ice in a solubilization medium containing 10% (v/v) glycerol, 20 mM Mops-KOH, pH 7.5, 1 mM *p*-aminobenzamidine, 2 mM DTT, 1.5 mM ITP, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5 μg mL^{-1} leupeptin, and 0.25 M KBr, and then centrifuged for 35 min at 110,000*g*. The supernatant was added with 375 μg mL^{-1} Brij 58. When necessary, the pellets were placed in resuspension medium with 1 mM $CaCl_2$ added.

CaM-Affinity Chromatography

Five-hundred microliters of CaM-agarose (catalog no. p-4385, Sigma) was transferred to a conical, 2-mL polypropylene tube and preequilibrated with solubilization medium with 0.5 mg mL^{-1} L-α-phosphatidylcholine and 375 μg mL^{-1} Brij 58 added. The solubilized PM proteins (approximately 1.2 mL) were applied and kept overnight under gentle rotation at 4°C. After a short centrifugation, the soluble phase (the unbound fraction) was aspirated and replaced with 1.2 mL of washing medium containing 10% (v/v) glycerol, 20 mM Mops-KOH, pH 7.5, 1 mM *p*-aminobenzamidine, 2 mM DTT, 100 μM $CaCl_2$, 100 μM $MgSO_4$, 5 μg mL^{-1} leupeptin, and 0.25 M KBr; a second wash was performed in the same medium in absence of $CaCl_2$ and $MgSO_4$. CaM-bound proteins were eluted in 1.2 mL of 10% (v/v) glycerol, 1 mM Mops-KOH, pH 7.5, 1 mM ITP, 375 μg mL^{-1} Brij 58, and 0.5 mg mL^{-1} L-α-phosphatidylcholine in the presence of 1 mM EGTA (twice) or 5 mM EDTA. The eluted fractions were added with stoichiometric $CaCl_2$ to neutralize EGTA or EDTA, and immediately used for assay of Ca^{2+}-ATPase activity or frozen in aliquots and kept at −80°C.

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

Unless otherwise specified, the hydrolytic activity of the PM Ca^{2+}-ATPase was measured as Ca^{2+}-dependent Mg-ITP hydrolysis (Carnelli et al., 1992). The assay medium contained 40 mM BTP-Hepes, pH 7.0, 50 mM KCl, 3 mM $MgSO_4$, 0.1 mM ammonium molybdate, 1 mM ITP, 5 μM carbonyl cyanide *p*-[trifluoromethoxy]phenylhydrazone, 5 μM A_{23187}, 1 μg mL^{-1} oligomycin, 5 mM $(NH_4)_2SO_4$, 0.1 mg mL^{-1} Brij 58, and 1 mM EGTA plus or minus $CaCl_2$ to give a free Ca^{2+} concentration of 50 μM (De Michelis et al., 1993). CaM was supplied at 20 μg mL^{-1}; incubation was performed at 25°C for 90 min. Ca^{2+}-ATPase activity was determined as the difference between the activity measured in presence of Ca^{2+} and that measured in its absence.

Treatment of the PM with FITC

The different fractions were diluted 20-fold with water to minimize interference by ITP (Rasi-Caldogno et al., 1995) and then incubated in 50 μM $CaCl_2$ and 20 mM BTP-Hepes, pH 7.0, in the presence of 5 μM FITC from a freshly prepared 75 μM solution in *N,N*-dimethylformamide. After incubation for 15 min at 25°C, the samples were precipitated in 10% (v/v) TCA for 2 h at 0°C, and centrifuged for 30 min at 80,000*g*. The pellets were washed with water, centrifuged again, and placed in resuspension medium.

Protein Assay

Protein was assayed according to the method of Markwell et al. (1978). The EDTA-eluted fraction was first precipitated with 10% (v/v) TCA as decribed above, to avoid interference by L-α-phosphatidylcholine.

SDS-PAGE and Western Analysis

SDS-PAGE was performed according to the method of Laemmli (1970). The different fractions were incubated for 5 min on ice in a cocktail of protease inhibitors (Rasi-Caldogno et al., 1995), and then solubilized for 60 min at 25°C in 4% SDS, 3% β-mercaptoethanol, 20% (v/v) glycerol, 1 mM EDTA, and 20 mM H_3PO_4 adjusted to pH 2.4 with Tris. Proteins from different fractions (0.2–80 μg per lane) were loaded onto gel (7.5% Tris-Gly gel with 4% stacking gel, catalog no. 161-0900, Bio-Rad). After electrophoresis, the gel was stained using the silver-impregnation method (catalog no. AG-5, Sigma) or blotted as described by Rasi-Caldogno et al. (1995). Immunodetection of FITC-labeled proteins (Rasi-Caldogno et al., 1995) was per-

formed with an anti-fluorescein rabbit IgG (H+L) fraction (catalog no. A-889, Molecular Probes, Sunnyvale, CA) and with a second antibody coupled to alkaline phosphatase (catalog no. A9919, Sigma). Immunodetection with an antiserum against the peptide encoded by PEA1 cDNA (kindly supplied by N.E. Hoffman, Carnegie Institution of Washington, Stanford, CA) was as described by Huang et al. (1993a). ^{125}I-CaM overlay was also as described previously (Rasi-Caldogno et al., 1995).

Statistics

Data are from one experiment representative of at least three experiments performed on PM Ca^{2+}-ATPase purified on three separate occasions. Assays of PM Ca^{2+}-ATPase activity were run with three replicates; SE of the assay did not exceed ± 4%.

RESULTS

Purification of the PM Ca^{2+}-ATPase by CaM-Agarose-Affinity Chromatography

Previous work had shown that the PM Ca^{2+}-ATPase is very sensitive to inactivation by detergents (Graf and Weiler, 1990; Kasai and Muto, 1991; Carnelli et al., 1992). In a first set of experiments we have thus compared the ability of different detergents to solubilize the Ca^{2+}-ATPase from PM purified from radish seedlings in active form. With Triton X-100 or 3-([cholamidopropyl]dimethylammonio-)1-propanesulfonate no more than 10% of the PM Ca^{2+}-ATPase activity could be recovered in the soluble fraction, even when phosphatidylcholine was added to the solubilization medium (Graf and Weiler, 1990; Hwang et al., 1997). Similar results were obtained with octylglucopyranoside, methylglucamide, and polyoxyethylene 8-myristyl ether (data not shown). Only solubilization with *n*-dodecyl β-D-maltoside (1:1 mg of detergent:mg of protein) yielded about 70% of the PM Ca^{2+}-ATPase activity in the soluble fraction (Table I); varying the protein and detergent concentration between 1 and 10 mg mL^{-1} or the addition of phosphatidylcholine to the solubilization medium had no major effect on Ca^{2+}-ATPase solubilization (data not shown).

Given the low concentration of Ca^{2+}-ATPase in the PM, for the purification of the enzyme by affinity chromatography with CaM-agarose, we chose to adopt a batch procedure that allows recovery of the eluted fractions in relatively small volumes (details are given in "Materials and Methods"). Preliminary experiments showed that no Ca^{2+}-ATPase activity could be recovered unless phosphatidylcholine was included in the elution medium (data not shown; see also Hwang et al., 1997).

Table I shows the results of a typical purification procedure. Upon overnight incubation of PM protein solubilized with *n*-dodecyl β-D-maltoside (4:4 mg of detergent:mg of protein) with CaM-agarose, the bulk of CaM-stimulated Ca^{2+}-ATPase activity bound to the matrix: the unbound fraction contained the bulk of protein and most of the CaM-independent Ca^{2+}-ATPase activity measured in the solubilized fraction, but stimulation by CaM of the Ca^{2+}-ATPase activity was virtually undetectable.

Subsequent washes of the matrix with decreasing concentrations of Ca^{2+} released only traces of Ca^{2+}-ATPase activity, which was virtually insensitive to CaM. Also, elution of CaM-bound proteins with 1 mM EGTA released very low amounts of Ca^{2+}-ATPase activity, also only slightly stimulated by CaM.

EDTA (5 mM) eluted a substantial amount of CaM-stimulated Ca^{2+}-ATPase activity: in 10 purifications performed independently, the activity in the EDTA-eluted fraction, measured in the presence of CaM, was 25 ± 2% of the loaded activity. The CaM-agarose purification procedure determined a marked increase of CaM stimulation of the PM Ca^{2+}-ATPase activity. In the experiment shown in Table I, CaM stimulation in the EDTA-eluted fraction was about 260% (compared with 120% in the solubilized PM),

Table I. *Purification of the PM Ca^{2+}-ATPase by CaM-agarose affinity chromatography*

PM proteins were solubilized with *n*-dodecyl β-D-maltoside (4:4 mg detergent mL^{-1}: mg protein mL^{-1}) and purified by CaM-agarose-affinity chromatography as described in "Materials and Methods." The first wash was performed in the presence of 100 μM $CaCl_2$ and 100 μM $MgSO_4$; the second one was performed in the absence of added divalent cations. Data in the brackets represent the percent stimulation by CaM (20 μg mL^{-1}). Results are from one experiment, which is representative of more than 10 experiments.

Fraction	Protein	Ca^{2+}-ATPase Activity					
		Total			Specific		
		−CaM	+CaM	ΔCaM	−CaM	+CaM	ΔCaM
	mg	*nmol Pi min^{-1}*			*μmol min^{-1} mg^{-1} protein*		
PM	5.7	96	260	164 (+171%)	0.017	0.045	0.028
Solubilized PM	4.1	90	200	110 (+122%)	0.022	0.049	0.027
CaM-agarose							
Unbound	3.6	80	90	10 (+12%)	0.022	0.025	0.003
Wash no. 1	ND[a]	11	12	1 (+9%)			
Wash no. 2	ND	4	4				
1 mM EGTA, eluate no. 1	ND	9	11	2 (+22%)			
1 mM EGTA, eluate no. 2	ND	9	12	3 (+33%)			
5 mM EDTA eluate	0.02	16	58	42 (+260%)	0.8 (×47)	2.9 (×65)	2.1 (×75)

[a] ND, Not determined.

but in some preparations CaM stimulation of the Ca^{2+}-ATPase in the EDTA-eluted fraction was up to 600% (see Fig. 1 and Table II). The source of this variability will be discussed below. The EDTA-eluted fraction contained only about 0.5% of the loaded proteins, so that it was 65-fold enriched in Ca^{2+}-ATPase activity (and 75-fold in CaM-dependent Ca^{2+}-ATPase activity) compared with the native PM.

In the experiment described in Table I, the PM Ca^{2+}-ATPase activity was assayed as Ca^{2+}-dependent ITPase activity, the procedure routinely used with native PM vesicles, to avoid interference due to the simultaneous activity of the much more abundant H^+-ATPase (Rasi-Caldogno et al., 1989; Carnelli et al., 1992). Table II shows that in the EDTA-eluted fraction the nucleoside triphosphatase activity measured in the absence of Ca^{2+} was also extremely low when ATP was supplied as a substrate, indicating that the fraction was devoid of H^+-ATPase activity.

Figure 1 shows the results of the electrophoretic separation of the EDTA-eluted fraction from four independent purification procedures. The EDTA-eluted fractions were highly enriched in a band of about 133 kD; two more bands at 180 and 120 kD were also visible. The intensity of the 120-kD band compared with the 133-kD band, was quite variable from experiment to experiment and was inversely related to the extent of CaM stimulation of Ca^{2+}-ATPase activity.

Identification of the PM Ca^{2+}-ATPase in the EDTA-Eluted Fraction

The molecular mass (133 kD) of the most abundant band in the EDTA-eluted fraction closely matches that previously reported for the intact Ca^{2+}-ATPase in native PM, which was identified by different methods (Rasi-Caldogno et al., 1995).

The Ca^{2+}-ATPase is the major protein labeled by CaM overlay of western blots of PM proteins of radish seedlings (Rasi-Caldogno et al., 1995). Figure 2A shows the CaM overlay of a western blot of the main fractions of the CaM-agarose purification procedure. ^{125}I-CaM heavily labeled a 133-kD band in the solubilized PM fraction (lane 1) and in the EDTA-eluted lane (lane 4); labeling was much weaker both in the fraction that did not bind to CaM-agarose (lane 2), and in the EGTA-eluted fraction (lane 3).

The PM Ca^{2+}-ATPase is extremely sensitive to inhibition by fluorescein derivatives (Giannini et al., 1987; Rasi-Caldogno et al., 1987, 1989, 1995; Olbe and Sommarin, 1991), which act as competitive inhibitors with the nucleoside triphosphates (De Michelis et al., 1993). Treatment of the PM with low concentrations of FITC selectively labels the Ca^{2+}-ATPase, which can be easily detected on western analysis with an anti-FITC antiserum (Rasi-Caldogno et al., 1995). Figure 2B shows the western blot of native PM and of the EDTA-eluted fraction, labeled with FITC under se-

Table II. *Substrate specificity of the partially purified PM Ca^{2+}-ATPase activity*

Assays were performed as described in "Materials and Methods," in the presence of 1 mM ATP or ITP; values in parentheses represent the Ca^{2+}-dependent activities.

Assay Conditions	Nucleoside Triphosphatase Activity	
	ATP	ITP
	nmol min^{-1} mg^{-1} protein	
$-Ca^{2+}$	0.27	0.25
$+Ca^{2+}$	0.70 (0.43)	0.64 (0.39)
$+Ca^{2+}$ + CaM	3.26 (2.99)	3.04 (2.79)

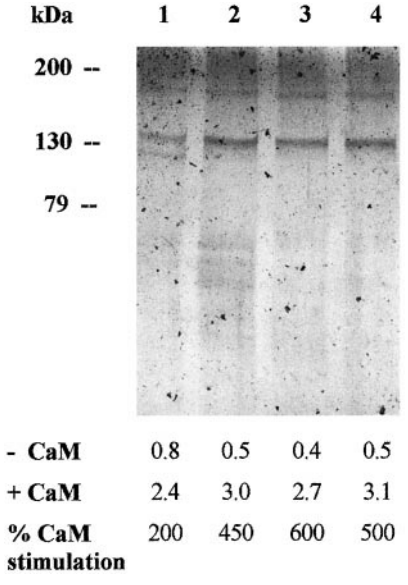

Figure 1. Silver stain of EDTA-eluted fractions from different purifications with different stimulation by CaM. Each lane was loaded with similar Ca^{2+}-ATPase activity (approximately 0.2 μg of proteins per lane). Values below the picture represent the Ca^{2+}-ATPase activities, expressed in micromoles per minute per milligram of protein.

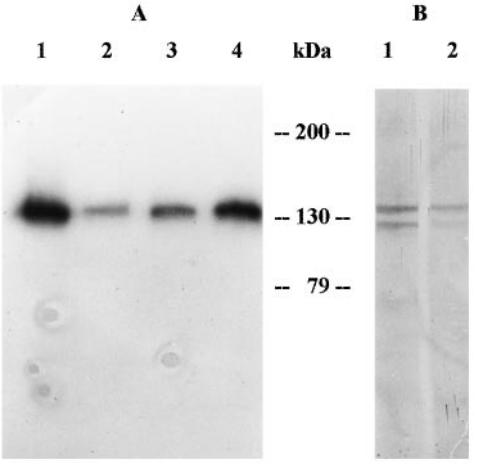

Figure 2. A, Labeling of the PM Ca^{2+}-ATPase of different fractions of the CaM-agarose purification procedure by ^{125}I-CaM. Proteins were from solubilized PM (lane 1), unbound fraction (lane 2), EGTA-eluted fraction (lane 3), and EDTA-eluted fraction (lane 4). Each lane contained protein solubilized from 10 μL of the relevant fraction. B, Labeling of the PM Ca^{2+}-ATPase of native PM (lane 1, 70 μg of proteins) and of the EDTA-eluted fraction (lane 2, 0.2 μg proteins) by FITC and immunodetection on western analysis with an anti-FITC antiserum.

lective conditions. In agreement with previous observations, FITC labeled two major bands of 133 and 120 kD in native PM (lane 1), which have been identified, respectively, as the intact Ca^{2+}-ATPase and a product of its proteolysis lacking the CaM-binding domain (Rasi-Caldogno et al., 1995). In the EDTA-eluted fraction (lane 2), FITC labeled the same two bands, but the signal was much stronger for the 133-kD protein. Conversely, in the fraction that did not bind to CaM-agarose, FITC labeling was much stronger for the lower-molecular-mass band (data not shown).

The first plant cDNA encoding a polypeptide with high homology with the PM Ca^{2+}-ATPase of mammals is *PEA1*, which encodes a putative Ca^{2+}-ATPase of the plastid envelope of *Arabidopsis thaliana* (Huang et al., 1993b). Figure 3 shows the western blot of the major fractions of our purification procedure labeled with an antiserum against a portion of the protein encoded by *PEA1* (kindly supplied by N.E. Hoffman). The antiserum labeled two bands of about 133 and 120 kD in all of the fractions tested. The 133-kD band was the more heavily labeled in the EDTA-eluted fraction (lane 4), whereas the fraction that did not bind to CaM-agarose (lane 3) was most enriched for the 120-kD protein.

DISCUSSION

In this paper we report the first purification, to our knowledge, of the Ca^{2+}-ATPase of the PM of plant cells. To achieve this goal we have used PM purified from germinating radish seedlings (Rasi-Caldogno et al., 1987, 1989, 1995).

The PM Ca^{2+}-ATPase tightly bound to the CaM-agarose matrix and was not substantially eluted by EGTA (even when the concentration was increased up to 10 mM, data not shown), which has been widely used to elute the plant CaM-stimulated Ca^{2+}-ATPases of endomembranes (Askerlund and Evans, 1992; Theodoulou et al., 1994; Askerlund, 1996; Hwang et al., 1997; Malmstrom et al., 1997). The tight binding of the PM Ca^{2+}-ATPase to the CaM-agarose matrix suggests a high affinity of the enzyme for CaM. This result is in agreement with the previously reported observation that in native PM the Ca^{2+}-ATPase is only slightly stimulated by exogenous CaM unless endogenous CaM is stripped by extensive washings with Ca^{2+} chelators (Williams et al., 1990; Rasi-Caldogno et al., 1993). In contrast, stimulation of the Ca^{2+}-ATPase activity by exogenous CaM is easily detected in endomembrane fractions (Hsieh et al., 1991; Askerlund and Evans, 1992; Bush and Wang, 1995; Askerlund, 1996; Askerlund and Sommarin, 1996; Hwang et al., 1997). The affinity for CaM may thus be a crucial difference between the PM Ca^{2+}-ATPase and Ca^{2+}-ATPases of endomembranes, and is worthy of further investigation.

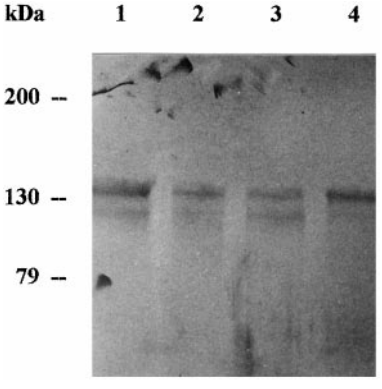

Figure 3. Immunodecoration of the main fractions of the CaM-agarose purification procedure, with an antiserum against a putative Ca^{2+}-ATPase of the *A. thaliana* plastid envelope. Lane 1 was loaded with proteins from native PM (70 μg), lane 2 with proteins from solubilized PM (32 μg), lane 3 with proteins from the unbound fraction (28 μg), and lane 4 with proteins from the EDTA-eluted fraction (0.23 μg).

Washing of the column with 5 mM EDTA eluted about 25% of the loaded activity and as much as 40% of the CaM-dependent activity. The EDTA-eluted fraction, which was about 70-fold enriched in Ca^{2+}-ATPase activity, was highly enriched in a 133-kD polypeptide, which was labeled by ^{125}I-CaM overlay, as well as by treatment with low concentrations of FITC. All of these characteristics closely match those determined for the PM Ca^{2+}-ATPase in native PM (Rasi-Caldogno et al., 1995). A second band of about 120 kD was present in variable amounts in the EDTA-eluted fraction. This band, which was enriched in the fraction that did not bind to CaM-agarose, and which was labeled by FITC but not by ^{125}I-CaM overlay, has been previously identified as a proteolytic product of the PM Ca^{2+}-ATPase (Rasi-Caldogno et al., 1995). Its presence in the purified Ca^{2+}-ATPase fraction probably reflects the activity of a co-purifying protease activity; accordingly, it was most abundant in the fractions in which the Ca^{2+}-ATPase activity was less stimulated by CaM (see Fig. 1B). Attempts to minimize proteolysis by means of various protease inhibitors have thus far been unsuccessful.

The yield and specific activity of the partially purified PM Ca^{2+}-ATPase agree with the best reports on the endomembrane- localized, CaM-stimulated Ca^{2+}-ATPase (Dieter and Marmè, 1981; Briars et al., 1988; Evans et al., 1989, 1992; Askerlund and Evans, 1992; Theodoulou et al., 1994; Askerlund, 1996; Hwang et al., 1997).

The PM Ca^{2+}-ATPase cross-reacts with an antiserum against a putative chloroplast envelope Ca^{2+}-ATPase of *A. thaliana* (Huang et al., 1993b), which is highly homologous to the tonoplast Ca^{2+}-ATPase of cauliflower (Malmstrom et al., 1997), as well as to mammalian PM Ca^{2+}-ATPases (Huang et al., 1993b). This result confirms that the same family of CaM-stimulated Ca^{2+}-ATPases is expressed in different membranes of plant cells (Evans, 1994; Askerlund and Sommarin, 1996).

Availability of a purified Ca^{2+}-ATPase of the PM is an essential prerequisite for cloning its gene. In fact, despite biochemical evidence indicating that CaM-stimulated Ca^{2+}-ATPases are the most abundant in plant cells, screening of cDNA libraries with heterologous probes yielded only clones of putative Ca^{2+}-ATPases homologous to the sarcoplasmic reticulum Ca^{2+}-ATPase of mammals (Perez-Prat et al., 1992; Wimmers et al., 1992). This is probably due

to the fact that heterologous probes mostly used for searching for P-type ATPases are based on the highly conserved nucleotide-binding site, which is likely to be variant in plant CaM-stimulated Ca^{2+}-ATPases. In fact, plant CaM-stimulated Ca^{2+}-ATPases differ from other P-type Ca^{2+}-ATPases for their ability to use GTP or ITP as alternative substrates and for their very high sensitivity to inhibition by fluorescein derivatives (Rasi-Caldogno et al., 1987, 1989; Williams et al., 1990; Olbe and Sommarin, 1991; Carnelli et al., 1992; De Michelis et al., 1993), which act as competitive inhibitors with respect to the nucleoside-triphospate substrate (De Michelis et al., 1993).

Only very recently (Malmstrom et al., 1997) has a gene encoding the cauliflower tonoplast Ca^{2+}-ATPase been identified using probes derived from the partial amino acid sequence of the purified enzyme: the deduced amino acid sequence shows high similarity to the mammalian PM Ca^{2+}-ATPase, but has the striking characteristic that the putative CaM-binding domain appears localized in the N-terminal rather than in the C-terminal domain (Malmstrom et al., 1997). The putative chloroplast envelope Ca^{2+}-ATPase, which is similar to the mammalian PM Ca^{2+}-ATPase, lacks the C-terminal CaM-binding domain and has an extended N-terminal domain (Huang et al., 1993b); PM-type Ca^{2+}-ATPases lacking the C-terminal CaM-binding domain have also been identified in the tonoplast of *Saccharomyces cerevisiae* (Cunningham and Fink, 1994) and *Dictyostelium discoideum* (Moniakis et al., 1995).

It will be interesting to find out whether displacement of the CaM-binding domain is a common feature of nonanimal PM-type Ca^{2+}-ATPases, or if it is typical of the endomembrane-localized members of this family. Unfortunately, preliminary attempts to sequence the 133-kD polypeptide in our purified PM Ca^{2+}-ATPase from radish seedlings have failed due to N-terminal blockage. Work is in progress to obtain partial amino acid sequences from tryptic fragments that are suitable for designing specific probes to isolate the PM Ca^{2+}-ATPase cDNA.

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