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Reconstitution and Characterization of a Calmodulin-Stimulated Ca^{2+}-Pumping ATPase Purified from *Brassica oleracea* L.[1]

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ABSTRACT

Purification and functional reconstitution of a calmodulin-stimulated Ca^{2+}-ATPase from cauliflower (*Brassica oleracea* L.) is described. Activity was purified about 120-fold from a microsomal fraction using calmodulin-affinity chromatography. The purified fraction showed a polypeptide at 115 kD, which formed a phosphorylated intermediate in the presence of Ca^{2+}, together with a few polypeptides with lower molecular masses that were not phosphorylated. The ATPase was reconstituted into liposomes by 3-([cholamidopropyl]-dimethylammonio-)1-propanesulfonate (CHAPS) dialysis. The proteoliposomes showed ATP-dependent Ca^{2+} uptake and ATPase activity, both of which were stimulated about 4-fold by calmodulin. Specific ATPase activity was about 5 μmol min^{-1} (mg protein)$^{-1}$, and the Ca^{2+}/ATP ratio was 0.1 to 0.5 when the ATPase was reconstituted with entrapped oxalate. The purified, reconstituted Ca^{2+}-ATPase was inhibited by vanadate and erythrosin B, but not by cyclopiazonic acid and thapsigargin. Activity was supported by ATP (100%) and GTP (50%) and had a pH optimum of about 7.0. The effect of monovalent and divalent cations (including Ca^{2+}) on activity is described. Assay of membranes purified by two-phase partitioning indicated that approximately 95% of the activity was associated with intracellular membranes, but only about 5% with plasma membranes. Sucrose gradient centrifugation suggests that the endoplasmic reticulum is the major cellular location of calmodulin-stimulated Ca^{2+}-pumping ATPase in *Brassica oleracea* inflorescences.

Calcium is an essential intracellular regulator in plant cells and is involved in metabolic and developmental regulation. Maintenance of a low free cytoplasmic concentration (about 0.1 μM) of Ca^{2+} ($[Ca^{2+}]_{cyt}$) is necessary for its function as a second messenger (18). This low $[Ca^{2+}]_{cyt}$ is maintained by the action of active Ca^{2+} transport systems assumed to be located at the plasma membrane, ER, and tonoplast (5, 12). Plant active Ca^{2+} transport systems fall into two categories, Ca^{2+}-pumping ATPases and Ca^{2+}/nH^+ antiporters. The former have been suggested to be located at the plasma membrane and ER, whereas the latter is located at the tonoplast (5, 12). CaM[4]-stimulated ATP-dependent Ca^{2+} pumps situated in the plasma membrane are believed to be of key importance for long-term regulation of $[Ca^{2+}]_{cyt}$ in animal cells (8, 9, 13, 27). A CaM-stimulated Ca^{2+}-ATPase is also present in plants and was partly purified from maize microsomes 11 years ago using CaM-affinity chromatography (11). This enzyme was later shown by Briars et al. (4, 12) to be a P-type ATPase with a molecular mass of about 140 kD, properties identical to those of the Ca^{2+}-ATPase in animal plasma membranes (8, 9, 13, 27). Antibodies against the erythrocyte Ca^{2+} pump cross-reacted with the purified maize enzyme, but no reaction could be detected in western blots of maize microsomes. Further characterization of the purified CaM-stimulated Ca^{2+}-ATPase from plants has not been carried out (possibly due to the high instability of this ATPase after solubilization), and its ability to pump Ca^{2+} has never been demonstrated. In the present article, we report on the functional reconstitution, characterization, and cellular location of a CaM-stimulated Ca^{2+}-pumping ATPase purified from cauliflower (*Brassica oleracea* L.) inflorescences.

MATERIALS AND METHODS

Plant Material

Cauliflower (*Brassica oleracea* L.) inflorescences were purchased locally.

Preparation of a Microsomal Membrane Fraction

Inflorescenses (130 g) were homogenized using a blender fitted with razor blades in 275 mL of 50 mM Mops-BTP, pH 7.5, 0.33 M sucrose, 5 mM Na_2-EDTA, 5 mM DTT, 0.2% (w/v) casein (boiled enzymic hydrolysate, Sigma N4517), 0.2% (w/v) BSA (Sigma A3294, protease free), 0.6% (w/v) PVP, 1 mM benzamidine-HCl, and 0.5 mM PMSF. The homogenate was filtered through a nylon cloth and centrifuged at 10,000*g* for 15 min. The supernatant was centrifuged at 40,000*g* for 1 h. The resulting pellet was suspended with a glass/Teflon

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[4] Abbreviations: CaM, calmodulin; BTP, 1,3-bis(tris[hydroxymethyl]methylamino)propane; CHAPS, 3-([cholamidopropyl]dimethylammonio-)1-propanesulfonate; cmc, critical micellar concentration; octyl glucoside, *n*-octyl-β-D-glucopyranoside; MEGA 8, octanoyl-*N*-methylglucamide.

homogenizer in buffer A (25 mM Mops-BTP, pH 7.5, and 0.33 M sucrose) supplemented with 0.5 M NaCl, 1 mM Na_2-EDTA, 5 mM DTT, and 0.5 mM PMSF to a final volume of 25 mL, and was again pelleted at 100,000*g* for 45 min. The final washed microsomal pellet was suspended to about 3 mL with buffer A plus 5 mM DTT and 0.5 mM PMSF, except when used as starting material for phase partition (see below). All operations were carried out at 0 to 4°C. The membranes were frozen in liquid N_2 and stored at −70°C for later use.

Solubilization of the CaM-Stimulated Ca^{2+}-ATPase and Chromatography

Washed microsomes (82 ± 29 mg of protein, average of five experiments) were solubilized in 22.5 mL of buffer A plus 0.5 M NaCl, 5 mM $CaCl_2$, 0.1% (w/v) phospholipid (Sigma P3644; type IV-S), 2 mM $MgCl_2$, 2 mM ATP, 40 μM leupeptin, 0.5 mM PMSF, and 2 mM DTT, 250 mg of Triton X-100 (*Surfact-Amps*, Pierce, Rockford, IL) with stirring for 10 min at 4°C. The unsolubilized material (15 ± 1 mg of protein) was pelleted at 100,000*g* for 45 min. CaM-affinity chromatography was carried out essentially as described by Penniston et al. (27). The supernatant containing the solubilized proteins (66 ± 20 mg of protein) was applied to a 5-mL column of CaM agarose (Sigma P4385) that had been washed with buffer A plus 0.5 M NaCl, 5 mM $CaCl_2$, 0.4 mM ATP, 0.4 mM $MgCl_2$, 0.05% phospholipid, 0.05% (v/v) Triton X-100, 0.5 mM PMSF, and 1 mM DTT. After application of the sample, about 150 mL of this medium was allowed to pass through the column. The flow rate was about 1 mL/min. When the purified ATPase was not to be used for reconstitution, about 50 mL of wash medium without $MgCl_2$ and ATP was passed through the column before elution. When the ATPase was to be reconstituted into liposomes, the wash medium was instead followed by 50 mL of buffer A plus 0.5 M NaCl, 5 mM $CaCl_2$, 1 mM CHAPS (Sigma C3023), 0.5 mM PMSF, and 1 mM DTT (no phospholipid). CaM-binding proteins were in each case eluted with 10 mM EGTA. Immediately after elution, 0.2 mL of 0.1 M $CaCl_2$ was added to each 1.4-mL fraction. The eluted fractions were assayed for protein and/or ATPase activity. Peak fractions were pooled, supplemented with 10 mM DTT, and placed on ice.

Reconstitution of CaM-Stimulated Ca^{2+} Uptake and ATPase Activity

A mixed phospholipid preparation (Sigma P3644, type IV-S) was further purified (17) and stored in chloroform:methanol (2:1, v/v) at −70°C. After removing the solvents on a rotary evaporator followed by lyophilization overnight, the phospholipids were suspended with glass beads under N_2 in 25 mM Mops-BTP, pH 7.2, and 0.33 M sucrose (buffer B) to a final concentration of 26 mg/mL. CHAPS (40 mM) was then added and the suspension sonicated to clarity on a bath sonicator under N_2. To initiate reconstitution, 1 part of the phospholipid/CHAPS suspension (approximately 3 mL) was added to 3 parts of column eluate containing the ATPase (approximately 0.3 mg of protein in 8–12 mL, Table I). The mixture was briefly vortexed, incubated on ice for 1 h, and dialyzed against 1 L of buffer B plus 1 mM DTT and 0.5 mM PMSF at 4°C during about 120 h with six changes. This long dialysis time was found to be necessary to produce sealed proteoliposomes with the dialysis tubing used (6.3 mm diameter cellulose tubing, mol wt cutoff 12,000–14,000; BDH Chemicals Ltd, Poole, UK). During the last two changes, 20 g of prewashed Amberlite XAD-2 (BDH Chemicals) was added to the dialysis bath to adsorb CHAPS. After dialysis, 10 mM DTT was added to the proteoliposomes, and they were either directly assayed for CaM-stimulated Ca^{2+} uptake and ATPase activity or stored under N_2 at 0 to 4°C for later use. Entrapment of oxalate inside proteoliposomes was achieved by adding 0.2 M K_2-oxalate to the protein-phospholipid-CHAPS suspension and dialyzing in the presence of 0.2 M K_2-oxalate after removal of the Ca-oxalate precipitate by centrifugation. In these experiments, the dialysis medium was replaced with dialysis medium plus 0.2 M KCl and 20 g L^{-1} Amberlite XAD-2 during the last two changes.

Two-Phase Partition

Plasma membranes were purified from a microsomal fraction by partitioning in an aqueous polymer two-phase system (36 g, final weight, of 6.2% [w/w] Dextran T 500, 6.2% [w/w] PEG 3350, 330 mM sucrose, 7.5 mM KCl, and 5 mM K-phosphate, pH 7.8) essentially as described in ref. 22. The microsomal fraction was obtained as described above, except that 90 g of inflorescences were used as starting material and the last wash was omitted. With the phase composition used, the plasma membranes partitioned in the upper phase, whereas the intracellular membranes partitioned at the interphase and into the lower phase. To increase the purity of the fractions, the initial upper phase was repartitioned twice with fresh lower phase and the initial lower phase was repartitioned once with fresh upper phase. The final upper (U_3 and U_3'; ref. 22) and lower (L_2) phases were diluted 4 and 10 times, respectively, with buffer A plus 1 mM Na_2-EDTA, 1 mM DTT, and 0.5 mM PMSF, and the membranes were pelleted at 100,000*g* for 45 min. A small amount of the microsomal fraction used as starting material was diluted about 20-fold and pelleted in the same way. The pellets obtained were suspended in buffer A plus 1 mM DTT and 0.5 mM PMSF. The membranes were snap frozen in liquid N_2 and stored at −70°C. For measurements of ATP-dependent Ca^{2+} uptake, the predominantly right-side-out plasma membranes were frozen and thawed about four times to produce a mixture of inside-out and right-side-out vesicles (26).

Sucrose Gradient Centrifugation

A 10,000*g* supernatant (obtained as described above except that BSA was omitted from the homogenization medium) was loaded on top of a cushion of gradient buffer (25 mM Mops-BTP, pH 7.2, 2 mM Na_2-EDTA, and 10 mM KCl) plus 1.7 M sucrose and centrifuged in a swing-out rotor at 79,000*g* for 30 min. The membranes at the interphase were collected and mixed with 2 volumes of gradient buffer plus 40 μM leupeptin. The diluted membranes (approximately 0.7 mg of protein in 0.7 mL) were applied to a continous sucrose gradient (0.6–1.7 M sucrose in 10 mL of gradient buffer) and

Table I. *Balance Sheet for Purification and Reconstitution of CaM-Stimulated ATPase and ATP-Dependent Ca^{2+} Uptake from Cauliflower Microsomes*

The yield and purification factors refer to CaM-stimulated activity. Total activities are expressed in μmol min^{-1} and specific activities (between parentheses) in μmol min^{-1} (mg protein)$^{-1}$. A typical experiment is shown.

	Total Protein	Total and (Specific) Activity			Purification	Yield
		−CaM	+0.5 μM CaM	CaM-stimulated		
	mg					
Microsomes	78					
ATPase		17.2 (0.221)	19.6 (0.251)	2.41 (0.031)	1	100
Ca^{2+} uptake		0.66 (0.0103)	1.91 (0.0298)	1.25 (0.0195)	1	100
Solubilized microsomes	78					
ATPase		21.6 (0.277)	23.7 (0.304)	2.11 (0.027)	0.87	88
Supernatant	68					
ATPase		14.1 (0.207)	16.1 (0.237)	2.04 (0.030)	0.97	85
Pooled CHAPS-eluate	0.43					
ATPase		1.10 (2.55)	1.82 (4.23)	0.72 (1.68)	54	30
Oxalate-loaded proteoliposomes	0.32					
ATPase		0.378 (1.18)	1.59 (4.98)	1.22 (3.80)	122	51
Ca^{2+} uptake		0.030 (0.096)	0.144 (0.458)	0.114 (0.362)	18.6	7.5

centrifuged in a swing-out rotor for 2 h at 100,000*g*. Fractions (0.75 mL) were collected from the bottom of the gradient with a peristaltic pump and were aliquoted and stored at −20°C until analysis. All operations were carried out at 0 to 4°C. Sucrose concentration was measured with an Abbe Model 60/ED refractometer (Bellingham & Stanley Ltd., Tunbridge Wells, UK).

Ca^{2+} Uptake

Ca^{2+} uptake was measured in a medium containing buffer B plus 100 mM KCl, 0.1% (w/v) BSA (Sigma A7030), 5 mM $MgCl_2$, 2.5 mM ATP, 50 μM $CaCl_2$ (0.3–0.6 Bq $^{45}CaCl_2$ $pmol^{-1}$), 1 to 5 mM DTT, 1 mM NaN_3, 0.1 mM Na_2-molybdate (standard assay medium), and 1 to 2 μg of reconstituted ATPase or 5 μg of membrane protein in 0.1 mL for 4 to 5 min (except during time course experiments) at 34°C. Modifications of the standard assay medium and additions of CaM (from bovine brain; Sigma P2277) were as indicated in the figure legends. The sample was preincubated with assay medium for 20 to 30 min prior to starting Ca^{2+} uptake by adding ATP. Controls without ATP were run in parallel. The reaction was stopped by addition of 0.6 mL of buffer B plus 1 mM EGTA, and aliquots were immediately filtered through 0.20-μm (reconstituted Ca^{2+} pump) or 0.45-μm (membranes) pore-size Whatman cellulose nitrate membrane filters.

After washing four times with 1 mL of buffer B plus 1 mM EGTA, the filters were dried and the amount of $^{45}Ca^{2+}$ was measured by scintillation counting. Counting efficiency was 80 to 95%. Free $[Ca^{2+}]$ was measured with a Ca^{2+}-specific electrode (Orion Research Inc., Boston, MA) calibrated with the Ca^{2+} buffers of Tsien and Rink (35). Free $[Ca^{2+}]$ in the absence of ATP was about 100 μM, even though only 50 μM $CaCl_2$ was added to the assay. This additional Ca^{2+} was due to contaminants in other components of the assay buffer (e.g. sucrose). The free $[Ca^{2+}]$ in the absence of ATP (and proteoliposomes/membranes) was used as an estimate for total Ca^{2+} when calculating specific Ca^{2+} uptake rates. This approximation is valid because the assay medium contained no anions with high affinity for Ca^{2+}. In the presence of 2.5 mM ATP, the free $[Ca^{2+}]$ was about 40 μM.

ATPase Assay

ATPase activity was measured under identical conditions to Ca^{2+} uptake, but for 20 to 30 min or as indicated in figure legends. Liberated phosphate was measured with a modified Baginski procedure (36). ATPase activity of fractions directly eluted from the column was measured under similar conditions after mixing 0.05 to 0.1 mL of eluate (after addition of $CaCl_2$) with an equal volume of buffer A supplemented with 50 mM KCl, 0.2% (w/v) BSA, 5 mM $MgCl_2$, and 2 mM DTT. The reaction was started by the addition of 2.5 mM ATP.

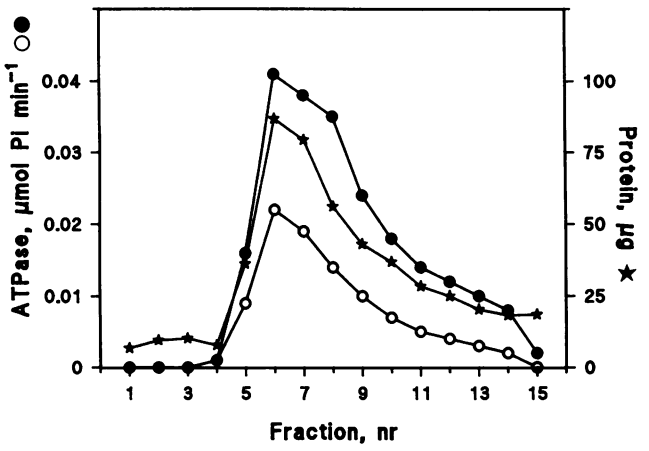

Figure 1. Elution profile from the CaM agarose column showing protein (★), and ATPase activity assayed in the absence (○) and presence (●) of 0.35 μM CaM. Fractions were collected immediately after addition of elution buffer. Fraction volume was 1.6 mL; ATPase was eluted in the presence of Triton X-100 and phospholipid.

Other Enzyme Activities

Antimycin A-insensitive NADH-Cyt *c* reductase activity was measured essentially as described in ref. 1. Cyt *c* oxidase was measured at 27°C in 1 mL of buffer B plus 25 mM KCl, 40 μM dithionite-reduced Cyt *c* (Sigma C7752), and 0.02% (w/v) Triton X-100. The reaction was started by the addition of membranes (40 μg of membrane protein or 25–50 μL of sucrose gradient fraction) and the oxidation of Cyt *c* recorded at 550 nM. An extinction coefficient of 19 mM^{-1} cm^{-1} for Cyt *c* was used. Pyrophosphatase activity was measured at 27°C in 0.1 mL of buffer A plus 7.5 mM $MgCl_2$, 100 mM KCl, 0.1% (w/v) BSA, and 15 μg of membrane protein. The reaction was started by the addition of 0.2 mM $Na_2P_2O_7$ and run for 30 min. Liberated phosphate and glucan synthase II were measured as described in ref. 36.

Phosphorylated Intermediate Formation

Phosphorylated intermediate formation was carried out in the presence of 20 mM Tris-HCl, pH 7.4, 12.5 μM $MgCl_2$, 50 μM $CaCl_2$, 15 μg of purified and reconstituted Ca^{2+}-ATPase, 37 kBq of [γ-^{32}P]ATP, and ±0.5 mM EGTA in a volume of 0.5 mL at 0°C for 15 s. The reaction was started with [γ-^{32}P]ATP and stopped by the addition of 2 mL of methanol that had been acidified with HCl (1 part 4 M HCl to 100 parts methanol). The precipitated protein was concentrated by subsequent additions of choroform and H_2O as described in ref. 28. Proteins were separated by SDS-PAGE in an acidic gel (5% acrylamide, pH 5.5) essentially as described in ref. 4. After Coomassie staining, the gel was dried and exposed for 18 d at −70°C with Fuji RX Medical X-ray film in the presence of a DuPont Cronex Lightning-plus intensifier screen. Precipitation with TCA gave a much stronger incorporation of ^{32}P (<12 h of exposure needed) than with the method described above, but TCA precipitation could not be used with the reconstituted Ca^{2+}-ATPase because the lipid also precipitated and interfered with SDS-PAGE.

Protein Analysis and Determination

Protein was measured with a modified Bradford procedure with BSA as the standard (33). When it was necessary to avoid interference with lipid, protein was first precipitated with methanol-chloroform-H_2O (28). In later experiments, the method of Kaplan and Pedersen (20) was used for quantitation of protein in the presence of large amounts of lipid. The two methods gave similar results, but the latter was more reproducible. SDS-PAGE was carried out on 7.5 to 15% gradient gels essentially according to Laemmli (23).

RESULTS

Solubilization and Purification of CaM-Stimulated Ca^{2+}-ATPase

Ca^{2+} uptake with washed cauliflower microsomes was stimulated about 200% by 0.5 μM CaM, whereas ATPase activity measured under identical conditions (i.e. in the absence of detergent) showed only about 14% stimulation (Table I). After solubilization of the membranes with Triton X-100 and application of the solubilized material to a CaM agarose column, a fraction (approximately 0.3 mg of protein) could be eluted with EGTA that showed a CaM-stimulated (50–100% stimulation) ATPase activity of 0.4 to 0.8 μmol Pi min^{-1} (mg protein)$^{-1}$ in the presence of a mixed phospholipid preparation (Fig. 1). Triton X-100 may have inhibited the isolated ATPase, because the activity was much higher when eluted in CHAPS (see below).

The fractions eluted from the CaM-affinity column were analyzed by SDS-PAGE. Coomassie-stained polypeptides were located at about 115, 52, 36, and 17 to 21 kD (Fig. 2B). Of these, only the 115-kD polypeptide formed a phosporylated intermediate after incubation with [γ-^{32}P]ATP. This phosphorylation was strongly stimulated by Ca^{2+} (Fig. 3).

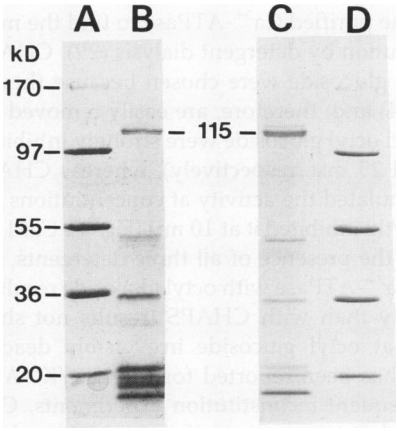

Figure 2. SDS-PAGE of fractions eluted from the CaM agarose column after washing with a medium containing Triton X-100 (B) and CHAPS (C). A and D, Standard molecular mass markers. The gels were stained with Coomassie brilliant blue R-250. Molecular mass standards (Boehringer Mannheim Combithek kit) in order of decreasing molecular mass were: α_2-macroglobulin, phosphorylase b, glutamate dehydrogenase, lactate dehydrogenase, trypsin inhibitor.

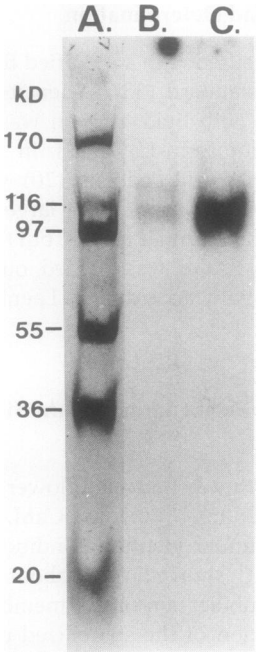

Figure 3. Analysis of the phosphorylated intermediate of the affinity-purified, CaM-stimulated Ca^{2+}-ATPase after SDS-PAGE in an acidic gel. Phosphorylation was carried out with [γ-^{32}P]ATP in B, the absence (+0.5 mM EGTA) or C, the presence of Ca^{2+}. A, Coomassie-stained standard molecular mass markers as in Figure 2, plus β-galactosidase (116 kD).

These results provide strong evidence that the 115-kD polypeptide represents the Ca^{2+}-ATPase (13).

Effects of Detergents on the Purified CaM-Stimulated Ca^{2+}-ATPase

The effects of three different detergents were tested on the activity of the purified Ca^{2+}-ATPase to find the most suitable for reconstitution by detergent dialysis (29). CHAPS, MEGA 8, and octyl glucoside were chosen because they all have a high cmc (24) and, therefore, are easily removed by dialysis. MEGA 8 and octyl glucoside were strongly inhibitory at their cmc (58 and 25 mM, respectively), whereas CHAPS (cmc = 6.5 mM) stimulated the activity at concentrations up to 5 mM and only partly inhibited it at 10 mM (Fig. 4). CaM stimulation was seen in the presence of all three detergents. Reconstitution of the Ca^{2+}-ATPase with octyl glucoside resulted in much lower activity than with CHAPS (results not shown). This indicated that octyl glucoside irreversibly deactivated the enzyme, as has been reported for the Na^+/K^+-ATPase (10). For all subsequent reconstitution experiments, CHAPS was used. We did not investigate if the inhibition by MEGA 8 was irreversible.

Reconstitution of CaM-Stimulated Ca^{2+} Uptake and ATPase Activity

For reconstitution, Triton X-100 was exchanged for CHAPS while the ATPase was still bound to the CaM agarose column. No activity could be detected in the eluate after change of detergent. The CHAPS wash resulted in some purification of the Ca^{2+}-ATPase as judged from the increase in intensity of the 115-kD polypeptide relative to the other polypeptides, as visualized by Coomassie-stained SDS-PAGE of the column eluate (cf. Fig. 2, B and C). The intensity of the low molecular mass polypeptides (17–21 kD) in particular was diminished by the CHAPS wash. Phospholipid, included in the buffers to stabilize the ATPase, was removed in the same step to be replaced by fresh phospholipid of higher purity during reconstitution. The ATPase activity was still high after elution in CHAPS-containing buffer with no lipid added (Table I), suggesting that the enzyme was not completely delipidated by this treatment (9, 27).

Insertion of the Ca^{2+}-ATPase into liposomes using the CHAPS-dialysis method resulted in a successful reconstitution of Ca^{2+} uptake (Fig. 5). To obtain maximal activities, it was necessary to preincubate the proteoliposomes for about 20 min in assay medium. If preincubation was omitted, a lag-phase was observed both for Ca^{2+} uptake and ATPase activity (results not shown). Preincubation with either Ca^{2+} plus Mg^{2+} or CaM has been necessary to achieve maximal rates of phosphorylation of the Ca^{2+}-ATPase from erythrocytes (13). Preincubation resulted in some binding of Ca^{2+} to the proteoliposomes (Fig. 5; time = 0). Subsequent addition of ATP resulted in a rapid accumulation of Ca^{2+}, reaching a steady state after about 15 min. All the accumulated Ca^{2+} could be released by the addition of the Ca^{2+} ionophore A23187. Measurement of ATPase activity under identical conditions as Ca^{2+} uptake gave a Ca^{2+}/ATP ratio of about 0.02 during the approximately linear, initial phase of Ca^{2+} uptake (Fig. 5). The ATPase activity was linear for at least 30 min. Inclusion of the Ca^{2+} ionophore A23187 in the assay medium stimulated the ATPase activity about 70 to 100% during the whole time course (Fig. 5).

When the Ca^{2+}-ATPase was reconstituted into liposomes

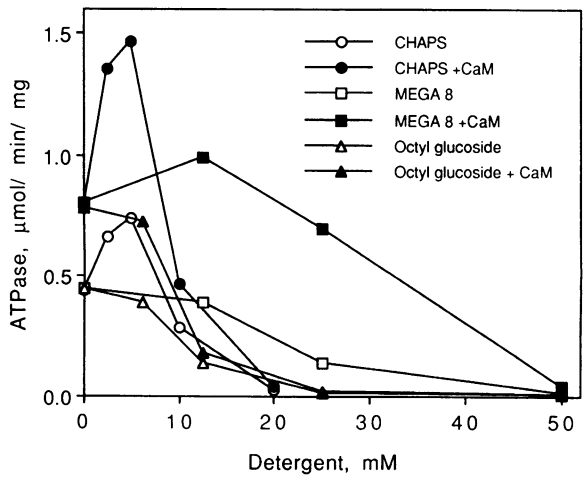

Figure 4. Effects of different detergents on the purified Ca^{2+}-ATPase in the absence (open symbols) and presence (filled symbols) of 0.35 μM CaM. The detergents tested were CHAPS (○, ●, cmc = 6.5 mM), MEGA 8 (□, ■, cmc = 58 mM), and octyl glucoside (△, ▲, cmc = 25 mM). The experiment was carried out with Ca^{2+}-ATPase eluted in the presence of Triton X-100 and phospholipid.

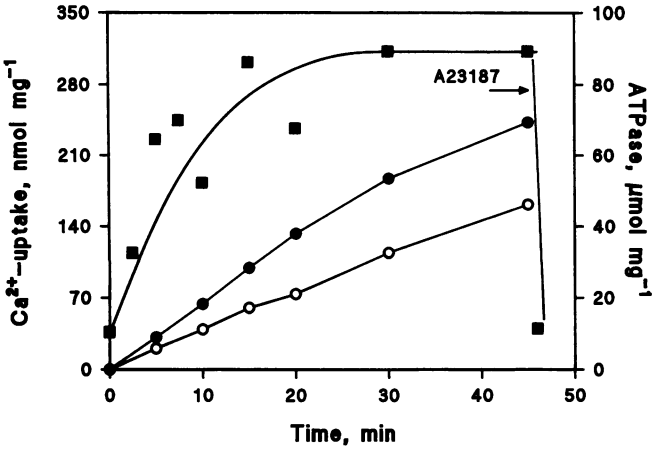

Figure 5. ATP-dependent Ca^{2+} uptake (■) and ATPase activity with purified and reconstituted Ca^{2+}-ATPase in the absence (○) and presence (●) of 5 μM of the Ca^{2+} ionophore A23187. The Ca^{2+}-ATPase was reconstituted into liposomes without oxalate. Accumulated Ca^{2+} could be released by 5 μM A23187. The standard assay medium was used except that the concentrations of KCl and $MgCl_2$ were 25 and 2.5 mM, respectively. CaM was present at a concentration of 0.5 μM.

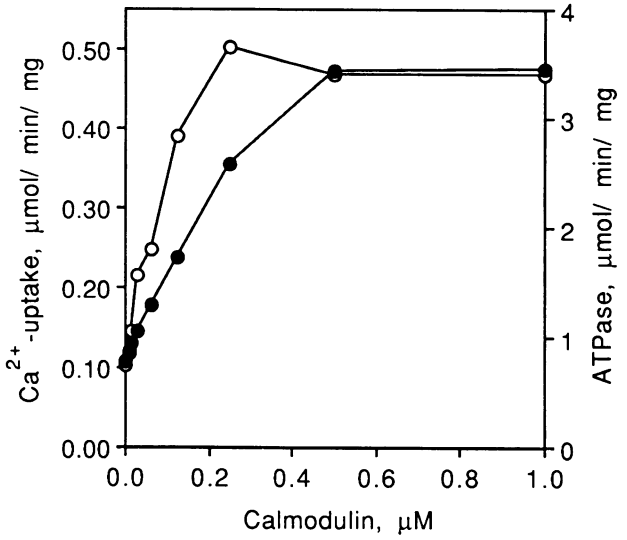

Figure 7. Effect of different concentrations of CaM on ATP-dependent Ca^{2+} uptake (○) and ATPase activity (●) with purified Ca^{2+}-ATPase reconstituted into liposomes. The proteoliposomes contained entrapped oxalate.

with entrapped oxalate, the initial rate of Ca^{2+} uptake was 10- to 20-fold higher than without entrapped Ca^{2+} chelator, whereas the ATPase activity was the same under both conditions (Fig. 6; the absolute rates in Figs. 5 and 6 should not be directly compared, however, because the concentrations of $MgCl_2$ and KCl were different in these two experiments; see figure legends). Thus, the Ca^{2+}/ATP ratio was increased to about 0.2 by entrapping oxalate in the proteoliposomes.

Both Ca^{2+} uptake and ATPase activity of the reconstituted Ca^{2+}-ATPase were stimulated about 300% by CaM (Figs. 6 and 7; Table I) in comparison with the 50 to 100% stimulation obtained before reconstitution (Figs. 1 and 4; Table I). To some extent, this difference may have been due to the much higher concentration of Ca^{2+} in the assay medium during measurements of ATPase activity with the enzyme directly eluted from the column (see below). The concentration of CaM needed for full stimulation of Ca^{2+} uptake was slightly lower than for full stimulation of ATPase activity (Fig. 7). This was also observed with microsomal membranes (data not shown).

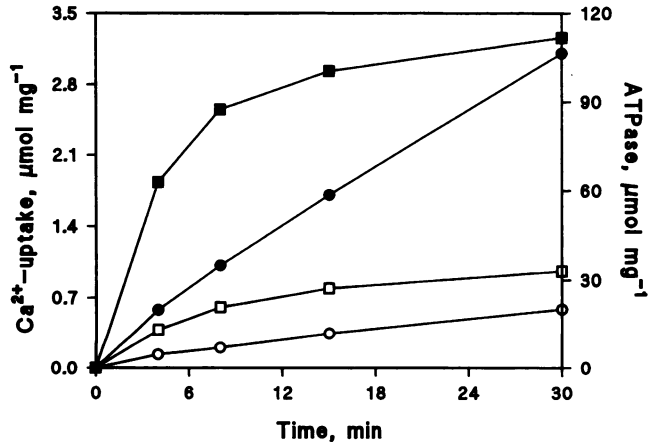

Figure 6. ATP-dependent Ca^{2+} uptake (□, ■) and ATPase activity (○, ●) with purified and reconstituted ATPase in the absence (open symbols) and presence (filled symbols) of 0.5 μM CaM. The Ca^{2+}-ATPase was reconstituted into liposomes that contained entrapped oxalate.

Further Characterization of the Reconstituted Ca^{2+}-ATPase

The ATPase activity of the reconstituted Ca^{2+}-ATPase was almost totally dependent on Ca^{2+} (Fig. 8). The half-maximal rate was reached at about 7 μM free [Ca^{2+}] both in the absence and presence of CaM. The ATPase activity was inhibited by concentrations of free [Ca^{2+}] above 1 mM, especially in the presence of CaM (Fig. 8). Both ATPase and ATP-dependent Ca^{2+} uptake by the reconstituted enzyme were completely dependent on Mg^{2+} and showed optima at about 5 mM $MgCl_2$ (Fig. 9).

The stimulation of the ATPase activity and ATP-dependent Ca^{2+} uptake activity of the purified and reconstituted Ca^{2+}-ATPase by KCl, KNO_3, and NaCl was 3-fold or higher (Fig. 10). The change in ionic strength was not controlled for and may account, in part, for the observed stimulation. The Ca^{2+} uptake showed an optimum at 100 mM KCl or NaCl, whereas the ATPase activity continued to increase above this concentration (Fig. 10). The ATPase activity and Ca^{2+} uptake of cauliflower microsomes showed no or very little stimulation by an increase in KCl from 25 to 100 mM (data not shown). This suggests that the activities obtained with membranes were less affected by ionic strength than those of the purified and reconstituted enzyme. Monovalent cations stimulate ATPase activity and Ca^{2+} uptake of erythrocyte ghosts by 30 to 100% (13), and ATP-dependent Ca^{2+} uptake by plant

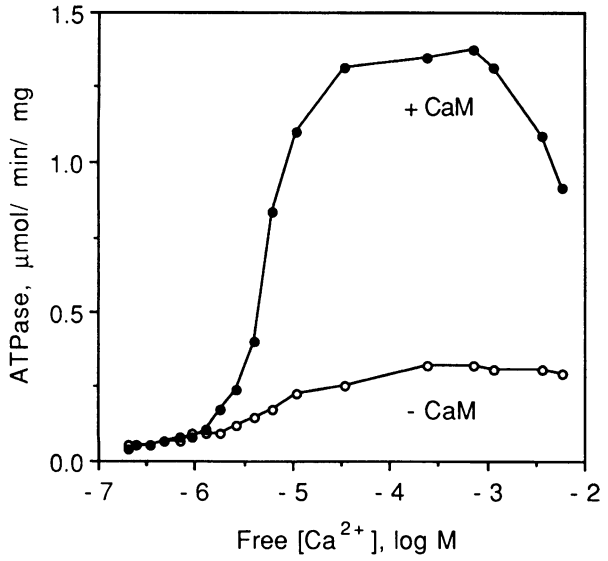

Figure 8. ATPase activity with purified and reconstituted Ca^{2+}-ATPase at different concentrations of free [Ca^{2+}] in the absence (open symbols) and presence (filled symbols) of 0.5 µM CaM. The assay medium was buffer B plus 2.5 mM $MgCl_2$, 25 mM KCl, 0.1% (w/v) BSA, 1 mM EGTA, 0.5 mM ATP, and varying amounts of $CaCl_2$. Free [Ca^{2+}] was measured with a Ca^{2+} electrode.

plasma membrane vesicles is stimulated to a similar degree (25).

ATP was the most effective nucleotide in supporting the Ca^{2+} uptake and NTPase activity of the reconstituted enzyme. Of the other nucleotides tested, GTP was the next most effective, exhibiting rates that were about 50% of those with ATP both in the presence (Fig. 11) and the absence of CaM (data not shown). Maximal activity with ATP was seen at pH 7.0. CaM stimulation was also strongest at this pH (Fig. 12).

Both ATPase activity and ATP-dependent Ca^{2+} uptake with the purified and reconstituted Ca^{2+}-ATPase were strongly inhibited by vanadate, showing that a P-type ATPase was responsible for the activities (Fig. 13). A clear inhibition of Ca^{2+} uptake was seen only when oxalate-loaded proteoliposomes were used. This may be explained by the ability of high intravesicular levels of free Ca^{2+} to protect against vanadate inhibiton (13). The ATPase activity was only slightly inhibited by thapsigargin (Table II), a potent and specific inhibitor of the ER (and not the plasma membrane or sarcoplasmic reticulum) vertebrate Ca^{2+}-ATPase (34). Cyclopiazonic acid (a specific inhibitor of the vertebrate sarcoplasmic reticulum Ca^{2+}-ATPase; ref. 32) had no effect on the cauliflower Ca^{2+}-ATPase (Table II). The lack of effect is in agreement with the observations of Hsieh et al. (19), who found no effect of cyclopiazonic acid on the CaM-stimulated component of ATP- or GTP-driven Ca^{2+} uptake with carrot cell membranes. Erythrosin B strongly inhibited the ATPase activity, with a K_i of 12 µM (Table II).

Cellular Location of the CaM-Stimulated Ca^{2+}-ATPase

Plasma membranes were prepared from the crude microsomal fraction by two-phase partitioning. The plasma membrane fraction represented about 12% of the total microsomal protein and was more than 4 times enriched in the plasma membrane marker glucan synthase II (Table III). The plasma membranes were almost completely depleted in Cyt *c* oxidase activity, a marker for the inner mitochondrial membrane. Pyrophosphatase activity (a tonoplast marker) and nonlatent, antimycin A-insensitive NADH-Cyt *c* reductase activity (a marker for the ER) were also strongly depleted in the plasma membranes: The specific activities of these markers were 19 and 6% of the activities in the microsomal fraction, respectively. The antimycin A-insensitive NADH-Cyt *c* reductase activity in both the microsomal and intracellular membrane fractions was inhibited by 0.015% (w/v) Triton X-100, whereas the activity in the plasma membrane fraction was strongly stimulated by the detergent (Table III). This latency of NADH-Cyt *c* reductase in plasma membrane vesicles is in agreement with earlier investigations (1, 36) and with the finding that a NADH-Cyt b_5 reductase-like enzyme with substrate binding sites on the cytoplasmic surface is present in plant plasma membranes (1).

In the absence of CaM, the specific ATP-dependent Ca^{2+} uptake was 100% higher in the plasma membranes compared to the microsomal fraction and the intracellular membrane fraction depleted in plasma membranes. In the presence of CaM, however, the activity was similar in all three fractions (Table III). Thus, CaM increased Ca^{2+} uptake about 200% in the microsomal and intracellular membrane fractions, but only about 50% in the plasma membrane fraction. Of total CaM-stimulated Ca^{2+} uptake, only about 6% was associated with the plasma membranes (Table III).

To investigate further the cellular location of CaM-stimulated Ca^{2+} uptake, microsomal membranes were separated on a continuous sucrose gradient (Fig. 14). To obtain a reasonable separation of the marker enzymes, it was necessary to

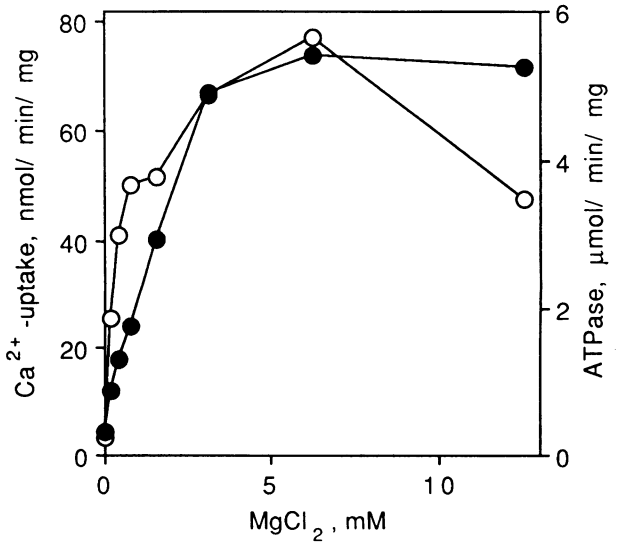

Figure 9. Effect of Mg^{2+} on ATP-dependent Ca^{2+} uptake and ATPase activity by purified and reconstituted Ca^{2+}-ATPase. CaM was present at a concentration of 0.5 µM. The Ca^{2+}-ATPase was reconstituted into liposomes without oxalate.

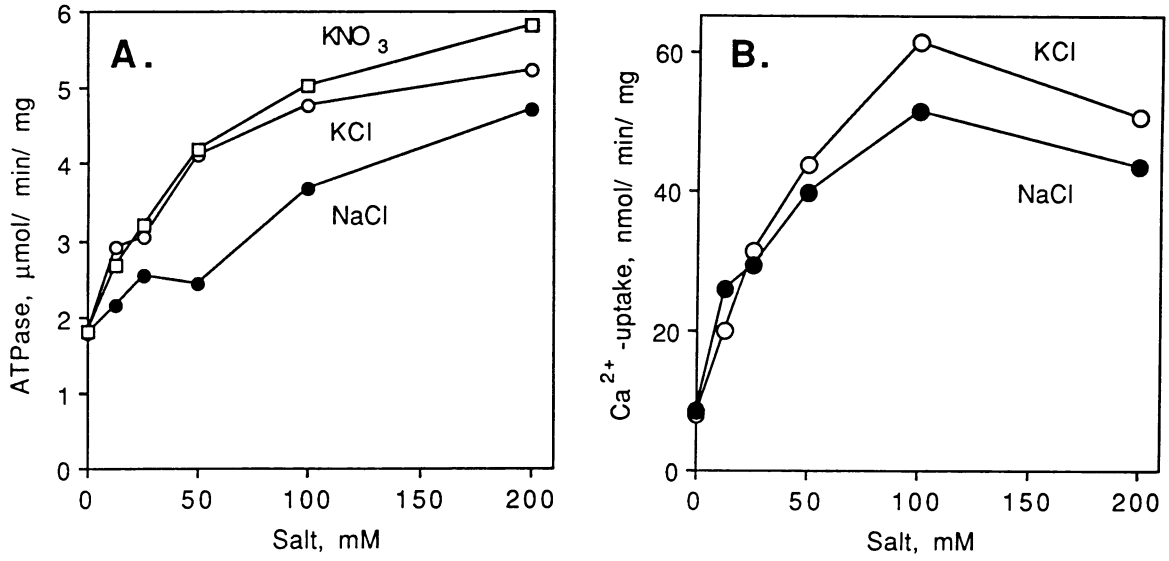

Figure 10. Effect of monovalent cations on ATPase activity (A) and ATP-dependent Ca^{2+} uptake (B) by purified and reconstituted Ca^{2+}-ATPase. CaM was present at a concentration of 0.5 µM. The Ca^{2+}-ATPase was reconstituted into liposomes without oxalate.

use nonfrozen membranes that had been concentrated on a sucrose cushion rather than by pelleting, and to include an excess of EDTA in all buffers. Ca^{2+} uptake in the absence of CaM was low and more or less evenly distributed across the gradient. In the presence of CaM, Ca^{2+} uptake was up to 6 times higher and showed a maximum at a density of about 1.12 g mL^{-1} (29% [w/w] sucrose), but a significant degree of activity was also present in both lighter and heavier fractions (Fig. 14, A and B). The peak of CaM-stimulated Ca^{2+} uptake coincided with the lighter of two peaks of antimycin A-insensitive NADH-Cyt *c* reductase activity, probably representing smooth ER, and was well separated from the glucan synthase II (plasma membrane marker) and Cyt *c* oxidase activities (Fig. 14, B and C). The heavier peak of NADH-Cyt *c* reductase is probably due to rough ER, plasma membrane, and other membranes containing this activity (Table III; refs. 1 and 36). CaM-stimulated Ca^{2+} uptake did not colocalize with pyrophosphatase activity (tonoplast marker; Fig. 14C). The protonophore FCCP (5 µM) had no significant effect on Ca^{2+} uptake in any of the fractions from the gradient, indicating that a Ca^{2+}/nH^+ antiporter (5, 12) was not responsible for a significant part of the activity in the microsomal fraction (data not shown).

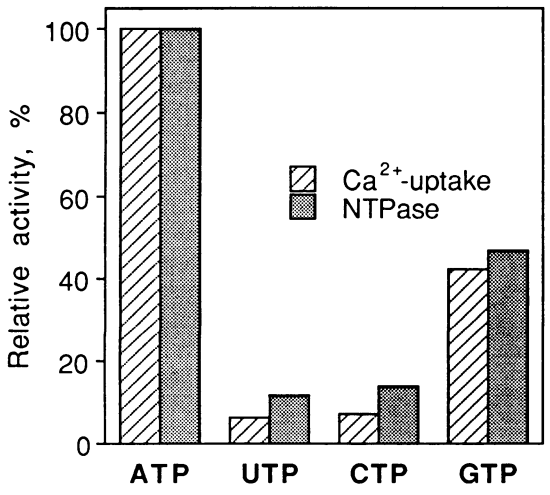

Figure 11. Relative NTPase (■) and Ca^{2+} uptake (▨) activity of the purified and reconstituted Ca^{2+}-ATPase with different nucleoside triphosphates (0.5 mM) in the presence of 0.5 µM CaM.

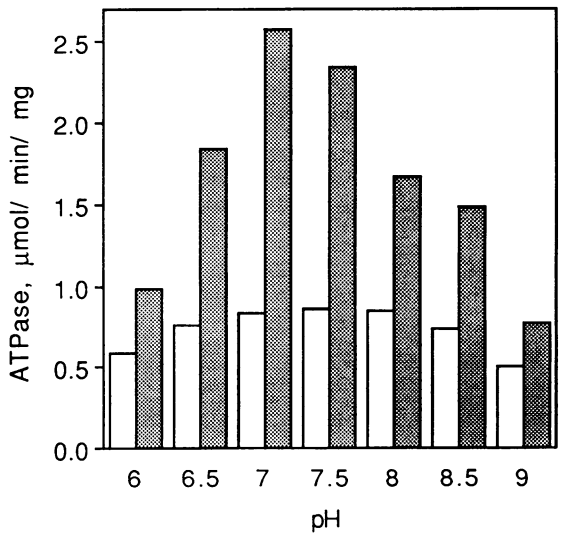

Figure 12. Effect of pH on the ATPase activity of the purified and reconstituted Ca^{2+}-ATPase in the absence (□) and presence (■) of 0.5 µM CaM. The Ca^{2+}-ATPase was reconstituted into liposomes without oxalate. Prior to addition to the assay medium, the ATPase was dialyzed against 2.5 mM Mops-BTP, pH 7.2, 0.33 M sucrose, and 2 mM DTT. The assay buffer was 25 mM Mes-BTP, pH 6 to 9. The assay medium included 5 µM Ca^{2+} ionophore A23187.

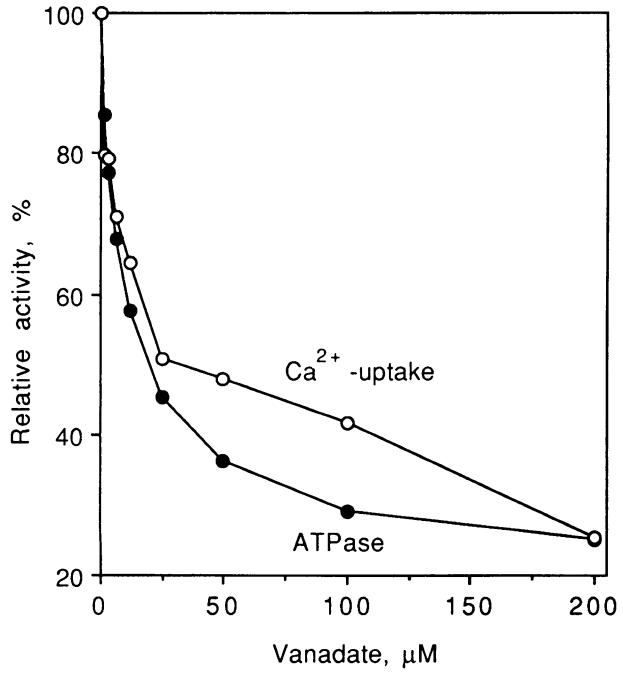

Figure 13. Effect of vanadate on ATPase activity and ATP-dependent Ca^{2+} uptake by the purified and reconstituted Ca^{2+}-ATPase. The proteoliposomes contained entrapped oxalate. CaM was present at a concentration of 0.5 μM.

DISCUSSION

The specific ATPase activity of the reconstituted cauliflower ATPase was high in the presence of CaM (approximately 5 μmol min^{-1} [mg protein]$^{-1}$, to be compared with 15–20 μmol min^{-1} [mg protein]$^{-1}$ reported for the purified CaM-stimulated ATPase from erythrocytes [13]). Calculation of the degree of purification of this activity cannot be based on the total ATPase activity of the membranes of origin because these contain other ATP hydrolyzers, including the plasma membrane H^+-ATPase. This is clearly illustrated by comparison of CaM stimulation of ATP hydrolysis in microsomes (14%; Table I) with CaM stimulation of ATP-dependent Ca^{2+} uptake (200%; Table I). The degree of purification can, however, be based on CaM-stimulated activities, both Ca^{2+} uptake and ATP hydrolysis (Table I). When CaM-stimulated ATPase activity with microsomes is compared with the CaM-stimulated ATPase activity after purification and reconstitution (conditions closest to those in the membrane), a purification factor of about 120 is achieved (Table I). CaM-stimulated Ca^{2+} uptake activity was purified only about 20-fold due to the 0.1 to 0.2 Ca^{2+}/ATP ratio (Table I).

The erythrocyte Ca^{2+}-ATPase can be purified to near homogeneity (approximately 250-fold enrichment of ATPase activity) with CaM-affinity chromatography and has a molecular mass of 130 to 140 kD (8, 9, 13, 27). CaM-stimulated ATPases in some other animal cells may have a slightly lower molecular mass (8). With cauliflower, the fraction eluted from the column showed a Coomassie-stained band (or possibly two bands very close together) at 115 kD together with a few polypeptides of lower molecular mass (Fig. 2). Only the 115-kD polypeptide formed a phosphorylated intermediate, and this formation was Ca^{2+} dependent (Fig. 3). This provides strong evidence that the 115-kD polypeptide represents the intact Ca^{2+}-ATPase, although the ATPase from maize microsomes has been reported to have a molecular mass of 140 kD (4, 12). Recently, Ca^{2+}-dependent formation of a 120-kD phosphoenzyme in membranes from carrot cells was demonstrated (19). The view that the purified cauliflower Ca^{2+}-ATPase was intact is further supported by the strong stimulation by CaM of ATPase activity and Ca^{2+} uptake after reconstitution (Figs. 6 and 7; Table I).

When reconstituted into liposomes, the Ca^{2+}-ATPase catalyzed an ATP-dependent accumulation of Ca^{2+} (Figs. 5 and 6). That Ca^{2+} was indeed taken up into the proteoliposomes by a transmembrane process is shown by the complete release

Table II. *Effect of Different Inhibitors on ATPase Activity with Purified and Reconstituted Ca^{2+}-ATPase*

Standard assay medium plus 0.5 μM CaM was used, except that the concentration of ATP was 0.5 mM in experiments with cyclopiazonic acid.

Inhibitor	ATPase Activity
	% of control
Thapsigargin	
0.1 μM	87
4.0 μM	78
Cyclopiazonic acid	
0.3 nmol (mg protein)$^{-1}$	96
10 nmol (mg protein)$^{-1}$	98
Erythrosin B	
12 μM	48
100 μM	10

Table III. *Distribution of CaM-Stimulated Ca^{2+} Uptake, Marker Enzyme Activities, and Total Protein between Plasma Membrane, Intracellular Membrane, and Microsomal Membrane Fractions*

The plasma membrane and intracellular membrane fractions were obtained by two-phase partitioning of the microsomal fraction and correspond to fractions designated $U_3 + U_3'$ and L_2 in ref. 22, respectively. All activities are specific and expressed in nmol min^{-1} (mg protein)$^{-1}$. Data are means ±SD from two different membrane preparations, except for antimycin A-insensitive NADH-Cyt c reductase and pyrophosphatase (one membrane preparation).

	Microsomal Membranes	Plasma Membranes	Intracellular Membranes
Total protein (mg)	53.3 ± 2.7	6.2 ± 0	43 ± 2.9
Ca^{2+} uptake			
−CaM	9.7 ± 1.6	16.6 ± 3.8	8.2 ± 1.6
+0.5 μM CaM	26.8 ± 2.3	24.5 ± 2.4	24.6 ± 2.6
CaM-stimulated	17.0 ± 0.7	8.0 ± 1.3	16.5 ± 1.0
Cyt c oxidase	183 ± 18.3	4.4 ± 0.4	298 ± 48.9
Antimycin A-insensitive NADH-Cyt c reductase			
−Triton X-100	330	19.4	370
+Triton X-100	252	141	252
Glucan synthase II	131 ± 39.4	549 ± 156	61.6 ± 17.6
Pyrophosphatase	82.2	15.4	83.3

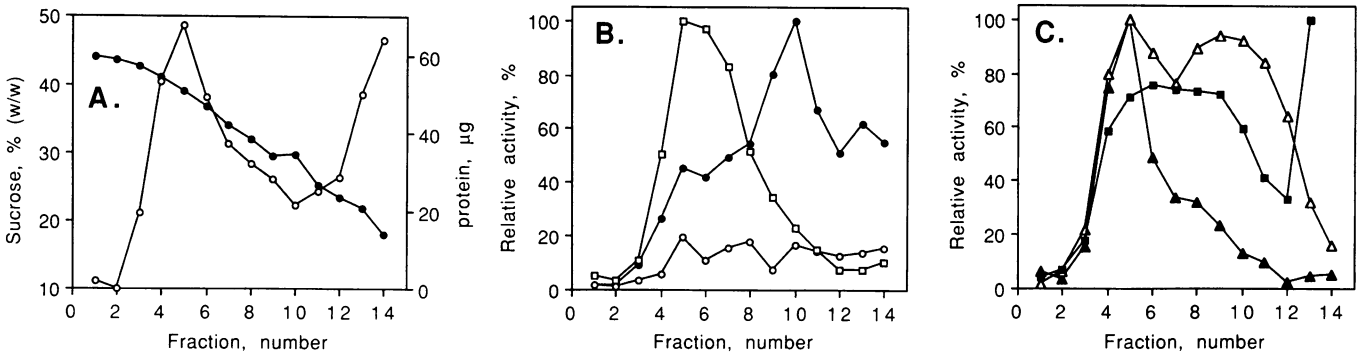

Figure 14. Distribution of different enzyme activities and protein after separation of microsomal membranes on a continuous sucrose gradient. A, ●, in percent (w/w) sucrose; O, in µg protein. B, O, ATP-dependent Ca^{2+} uptake without CaM (100%= 0.49 nmol min^{-1}); ●, ATP-dependent Ca^{2+} uptake with 0.5 µM CaM (100%= 0.49 nmol min^{-1}); □, glucan synthase II (100%= 4.0 nmol min^{-1}). C, ■, pyrophosphatase activity (100%= 12 nmol min^{-1}); ▲, Cyt *c* oxidase (100%= 61 nmol min^{-1}); Δ, antimycin A-insensitive NADH-Cyt *c* reductase (100%= 21 nmol min^{-1}; measured in the absence of Triton X-100).

of Ca^{2+} by the Ca^{2+} ionophore A23187 (Fig. 5). A direct comparison of Ca^{2+} uptake and ATPase activity using proteoliposomes without entrapped Ca^{2+} chelator gave a Ca^{2+}/ATP ratio of about 0.02 (Fig. 5). This low ratio and the fact that the ATPase activity failed to reach a plateau as the uptake of Ca^{2+} progressed with time (Fig. 5) indicated that the proteoliposomes were permeable to Ca^{2+}. Consequently, the Ca^{2+} ionophore A23187 stimulated the ATPase activity only 70 to 100% compared to 10-fold with the reconstituted erythrocyte ATPase (9). Entrapment of oxalate inside the proteoliposomes increased the rate of Ca^{2+} uptake 10- to 20-fold without affecting the ATPase activity and thus increased the coupling ratio to about 0.2 (Fig. 6; Table I). In a few experiments where a lower concentration of ATP than the standard 2.5 mM was used, coupling ratios as high as 0.5 were obtained (data not shown). This should be compared with the Ca^{2+}/ATP ratio of 0.63 for the CaM-stimulated activity in cauliflower microsomes (Table I) and a maximal Ca^{2+}/ATP ratio of 0.9 to 2.1 for the purified and reconstituted erythrocyte Ca^{2+}-ATPase (8, 9, 13). The highest coupling ratios with the erythrocyte ATPase have been obtained after reconstitution in "asolectin," a crude lipid mixture that produces highly sealed liposomes (9, 27). We used a slightly higher purity of lipid that may have resulted in more leaky liposomes. Residual CHAPS may also have caused the leakiness of the proteoliposomes to Ca^{2+} in the absence of entrapped oxalate.

Both Ca^{2+} uptake and ATPase activity measured with the reconstituted Ca^{2+}-ATPase were stimulated about 300% by CaM (Fig. 7; Table I). The erythrocyte Ca^{2+}-ATPase only shows such a strong stimulation by CaM in the presence of pure phosphatidylcholine. In the presence of acidic phospholipids, e.g. after reconstitution into "asolectin," the erythrocyte ATPase is fully stimulated (i.e. shows no stimulation by CaM; 8, 9, 13, 27). The phospholipid mixture used here is relatively crude (approximately 40% phosphatidylcholine, as supplied by Sigma Chemical Co.; the further purification carried out mainly removes contaminating protein, Ca^{2+}, and oxidation products). The strong CaM stimulation seen with the reconstituted cauliflower ATPase (Fig. 7) is therefore surprising.

The K_m [Ca^{2+}] for ATPase activity with the purified and reconstituted ATPase was about 7 µM in both the presence and absence of CaM (Fig. 8). This is different from the purified and reconstituted Ca^{2+}-ATPase from animal plasma membranes, which shows a significantly higher affinity for Ca^{2+} in the presence of CaM [K_m (Ca^{2+}) ≈ 1.5 µM; ref. 13] than in its absence [K_m (Ca^{2+}) ≈ 20 µM]. The ATPase activity was inhibited by concentrations of free [Ca^{2+}] above 1 mM (Fig. 8), resembling the case in animals (9). The properties of the cauliflower ATPase may have changed during purification or after reconstitution so that CaM can no longer increase its affinity for Ca^{2+}. Reported K_m (Ca^{2+}) for Ca^{2+}-dependent ATPase activity and ATP-dependent Ca^{2+} uptake range between 0.07 and 6 µM for both plant plasma membrane vesicles and ER (5, 7, 12, 14–16, 25, 30).

The cellular location of the CaM-stimulated Ca^{2+} pump in plants has long been a matter of controversy (5, 12). The analogy with animals would suggest that the activity should be present in the plasma membrane only, and both this and several other investigations indicate that the plant plasma membrane may contain a CaM-stimulated Ca^{2+} pump (Table III; 5, 12, 30, 31). In other studies, no CaM stimulation of plasma membrane ATPase activity or Ca^{2+} uptake has been found (15, 16, 21). In the present work, however, we clearly show that the major part of the ATP-dependent CaM-stimulated Ca^{2+} uptake is not in the plasma membrane, because only about 6% of the total activity was associated with the plasma membrane fraction and the remaining part with the intracellular membrane fraction (Table III).

After separation of the microsomal membranes on a continuous sucrose gradient, the peak of CaM-stimulated Ca^{2+} uptake coincided with the lighter of two peaks of antimycin A-insensitive NADH-Cyt *c* reductase activity at a density of 1.12 g mL^{-1} (the approximate density of smooth ER), suggesting that ER is the major cellular location of the CaM-stimulated Ca^{2+}-ATPase in cauliflower inflorescences (Fig. 14, B and C). The ER also seemed to be the location of CaM-stimulated Ca^{2+}-ATPase activity and Ca^{2+} uptake in membranes from maize coleoptiles (3) and carrot cells (19). In other studies of Ca^{2+} uptake and ATP hydrolysis with frac-

tions enriched in ER, no effect of CaM was reported (7). Possibly, the CaM-stimulated ATPase is present only in certain tissues or species, or CaM stimulation may be masked by high levels of endogenous CaM in membranes (5).

Although the Ca^{2+} pump from cauliflower inflorescences is CaM stimulated, it shows several properties that are different from the CaM-stimulated Ca^{2+} pump in animal plasma membranes and that are more or less similar to those of the animal endoplasmic/sarcoplasmic-type Ca^{2+} pump, which is not directly regulated by CaM. First, the major part of the activity is not located in the plasma membrane, but probably in the ER. Second, the cauliflower Ca^{2+}-ATPase has a slightly lower molecular mass (115 kD; Figs. 2 and 3) than the CaM-stimulated animal Ca^{2+}-ATPase, which has a molecular mass of 130 to 140 kD in most cells (8, 9, 13, 27). The indication of a double band seen with the cauliflower preparations may be due to the presence of two or more isoforms of the enzyme. Third, the cauliflower ATPase is much less specific for ATP (Fig. 11) than the CaM-stimulated Ca^{2+}-ATPase in animals, which uses only ATP as an energy donor (13). Ca^{2+} uptake with plant plasma membrane vesicles and ER from garden cress roots shows a similar specificity for ATP, as shown here for the purified Ca^{2+}-ATPase, with GTP (and ITP) exhibiting rates that are 25 to 50% of those with ATP (6, 15, 16, 25, 30), whereas the Ca^{2+}-ATPase activity in red beet ER was reported to use ATP only (14). Fourth, the cauliflower Ca^{2+}-ATPase showed a relatively low sensitivity to erythrosin B (K_i = 12 μM; Table II) in comparison to the erythrocyte ATPase (K_i = 70 nM; ref. 13 and refs. therein). This was not a purification artifact because ATP-dependent Ca^{2+} uptake by a fraction enriched in ER (obtained by sucrose gradient centrifugation) was similarly affected to the purified Ca^{2+}-ATPase (data not shown). In contrast, Ca^{2+} uptake with the plasma membrane fraction (obtained by two-phase partitioning) showed a much higher sensitivity to erythrosin B (K_i = 0.6 μM). This difference in sensitivity to erythrosin B may represent a fundamental difference in the ATP binding site(s) between plant ER and plasma membrane Ca^{2+} pumps (13). A monoclonal antibody raised against the erythrocyte Ca^{2+}-ATPase (5F10, kindly supplied by Prof. J. T. Penniston, Mayo Foundation, Rochester, MN) gave no significant reaction with intracellular and plasma membrane fractions from cauliflower in western blots (with the intracellular membrane fraction, two polypeptides, at 14 and 16 kD, were recognized; data not shown), even though it shows cross-reactivity with different plasma membrane Ca^{2+} pumps in animals (2, and refs. therein).

In summary, we have achieved the first reconstitution of a purified plant CaM-stimulated Ca^{2+}-pumping (Ca^{2+} + Mg^{2+})-ATPase. The molecular mass of this ATPase was determined to be 115 kD, and the enzyme was mainly confined to a light intracellular membrane fraction, probably the ER, in *Brassica oleracea* L.

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