Phytochemistry, Vol. 26, No. 8, pp. 2191–2196, 1987.
Printed in Great Britain.

0031-9422/87 $3.00 + 0.00

A HIGH-AFFINITY ($Ca^{2+} + Mg^{2+}$)-ATPASE FROM *ZEA MAYS* MICROSOMES*

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(*Revised received* 6 *January* 1987)

Key Word Index—*Zea mays*; Gramineae; maize; ATPase; Ca^{2+}.

Abstract—By assaying at low ATP concentrations without added Mg^{2+}, we have shown that microsomal membranes, prepared from *Zea mays* seedling shoots, contain a ($Ca^{2+} + Mg^{2+}$)-ATPase. The Mg^{2+} requirement of the enzyme is met by traces of Mg^{2+} present in the enzyme and substrate preparations. The enzyme is half-maximally activated at 0.2 μM Ca^{2+}. The enzyme has been solubilized and partially purified. The estimated M_r is $105 \pm 6 \times 10^3$, and the pH optimum is 6.5. It has a broad substrate specificity, hydrolysing nucleotide triphosphates with rates in the order ATP > UTP > CTP > GTP. Activity on *p*-nitrophenylphosphate is negligible. The enzyme is compared with a similar enzyme from animal cells.

INTRODUCTION

Plants contain a variety of enzymes capable of hydrolysing ATP [1]. They differ in substrate specificity, in sensitivity to various inhibitors and to activation by metal ions; they may be soluble or bound to various membranes, and their distribution among cell fractions may depend strongly on the experimental conditions [2]. With so many variables, it is important to be able to identify enzymic activities with characterized proteins. A great deal of progress has been made in the solubilization and characterization of H^+-translocating ATPases [3], but much less is known about other ATPases. A recurrent question concerns whether a particular activity is due to an ATPase or to a non-specific phosphatase (e.g. [2]). A necessary step in sorting out the many ATP-hydrolysing activities is purification and characterization of the proteins involved. This paper reports partial purification and characterization of a ($Ca^{2+} + Mg^{2+}$)-dependent ATPase and comparison of its properties with other Ca^{2+}-ATPases.

RESULTS

Attempts to study ($Ca^{2+} + Mg^{2+}$)-ATPase activity in plant membrane preparations are complicated by the presence of Mg^{2+}-dependent, K^+-stimulated ATPase in quantities that obscure the activity of the ($Ca^{2+} + Mg^{2+}$)-ATPase. To circumvent this problem, we assayed for ($Ca^{2+} + Mg^{2+}$)-ATPase under conditions that minimize the activity of the ($Mg^{2+} + K^+$)-ATPase i.e. low levels of ATP (100 μM) and no added Mg^{2+} or K^+ at pH 6.5. A Ca^{2+}-stimulated activity dependent upon endogenous Mg^{2+} can be detected under these conditions. It may also be important that no chelating agents are used in the isolation of the membranes.

Distribution of the ($Ca^{2+} + Mg^{2+}$)-*ATPase on a continuous sucrose-density gradient*

The 80000 *g* microsome fraction was centrifuged on a 20–50% sucrose density gradient (Fig. 1). The major peak of Ca^{2+}-stimulated ATPase activity is centred at 37% sucrose (1.16 g/cm^3), with a smaller peak at 42% sucrose (1.19 g/cm^3), corresponding to the reported densities of plasma membranes and mitochondria, respectively [4]. ($Mg^{2+} + K^+$)-ATPase activity paralleled the ($Ca^{2+} + Mg^{2+}$)-ATPase activity (data not shown). Fractions of densities 1.14–1.19 g/cm^3 were pooled. The endogenous

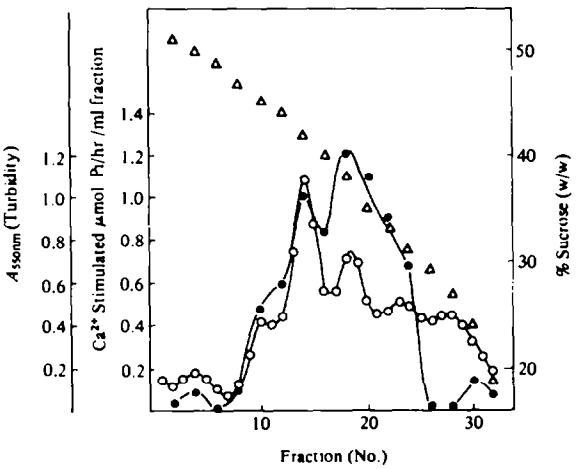

Fig. 1. Sucrose density-gradient profile of the 80000 *g* microsome pellet. ●——●, Ca^{2+}-stimulated ATPase activity; ○——○, membrane bands (scattering at 550 nm); △——△, sucrose concentrations. Each fraction is 32 drops.

*Journal Paper No. J-12363 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. 2560.

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Ca^{2+} and Mg^{2+} concentrations in assay mixtures containing this membrane preparation were 12.0 μM and 14.8 μM, respectively.

Properties of membrane-bound enzyme

In the absence of added divalent metal ion, four nucleoside triphosphates and *p*NPP were hydrolysed at similar rates (Table 1). Stimulation by added Ca^{2+} was nearly twofold with ATP as the substrate and decreased in the order UTP, GTP, CTP, *p*NPP.

The ATPase activity of the membranes without added divalent metal ions was relatively high, and the stimulation by added Ca^{2+} saturated at 50–100 μM at all ATP concentrations tested (Table 2). The conditions adopted for the standard assay included 0.1 mM ATP and 0.1 mM Ca^{2+}. The activity under these conditions was linear with time for at least one hr. The pH profile peaks at pH 6.5, with a smaller peak at pH 8.0 (Fig. 2). The effects of various inhibitors are shown in Table 3. Oligomycin had no effect, and sodium azide no consistent effect, suggesting that mitochondrial ATPase was not contributing to the observed activity. DCCD and DES had little effect on the basal activity and inhibited the Ca^{2+}-stimulation. Sodium molybdate and sodium vanadate inhibited the basal activity while having no effect on the Ca^{2+}-stimulated activity, suggesting that at least a portion of the basal activity was due to acid phosphatase but that the Ca^{2+}-stimulation was not. In a separate experiment, 50 μM fluphenazine (a calmodulin inhibitor) inhibited the Ca^{2+}-stimulation by 60% while having no effect on the basal activity.

To investigate the effects of Ca^{2+} and Mg^{2+} on ATPase activity, EGTA and CDTA (*trans*-cyclohexane-1,2-diamine-*N*,*N*,*N*′,*N*′-tetraacetic acid) were used as metal ion-buffering agents. Calculations of free Ca^{2+} concentrations in assay mixtures was performed by using variations of the equations and methods described in ref. [5]. The endogenous levels of Mg^{2+} and Ca^{2+} were incorporated into the calculations, as was the chelation by ATP. The ATPase activity of the membranes as a function of Ca^{2+} concentration in the presence of EGTA, which has low affinity for Mg^{2+}, is shown in Fig. 3. Half-maximal activation was found at 0.2 μM Ca^{2+}, comparable to Ca^{2+} levels in plant cells [6, 7]. When CDTA, which has high, nearly equal affinity for both Ca^{2+} and Mg^{2+}, was used (Fig. 3), much higher Ca^{2+} concentrations were required for activation. This suggested that, at low Ca^{2+} concentrations, the CDTA was binding the endogenous Mg^{2+} in the assay mixtures, but when the Ca^{2+} concentration was sufficiently high, Mg^{2+} was released and activated the enzyme. A Hill plot of the EGTA-buffered Ca^{2+} profile had a slope of 1.08, indicating that Ca^{2+} binding was not cooperative.

Table 1. Substrate specificity of the membrane bound (Ca^{2+} + Mg^{2+})-ATPase

	Enzymatic activity (μmol. Pi/hr/mg protein)		
	Addition to assay		
Substrate*	200 μM EGTA	100 μM Ca^{2+}	Ca^{2+} stim.
ATP	1.31	3.26	1.95
GTP	1.20	2.56	1.36
CTP	0.93	2.02	1.09
UTP	0.89	2.64	1.75
*p*NPP	1.09	1.54	0.45

*All substrates 100 μM. *p*NPP = *p*-nitrophenyl phosphate.

Table 2. Stimulation of ATP hydrolysis by Ca^{2+} at various ATP concentrations

	ATP hydrolysis (μmol. Pi/hr/mg protein)					
	Added Ca^{2+}, μM					
ATP (mM)	0	50	100	200	500	1000
0.1	176	436	461	452	472	517
0.2	367	550	646	646	660	698
0.5	642	846	928	957	837	872
1.0	982	1150	1179	1226	1239	1166
3.0	1799	1932	1926	1902	1981	1960

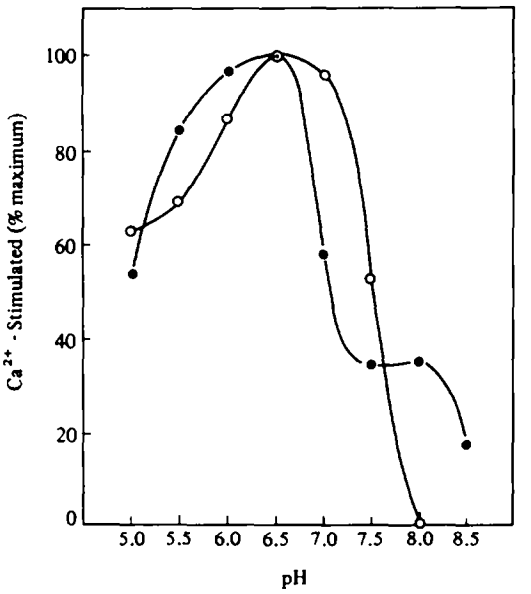

Fig. 2. pH profiles of (Ca^{2+} + Mg^{2+})-ATPase before and after solubilization. ●——●, Membrane-bound activity; ○——○, soluble enzyme. Buffer: 10 mM Hepes-imidazole.

Solubilization and purification

Membranes isolated by centrifugation in a two-step density gradient were extracted with detergent plus KCl, and the soluble material was dialysed and applied to a hexyl-agarose column (Fig. 4). A Ca^{2+}-ATPase activity eluted with low-salt buffer was of low specific activity and was accompanied by a large amount of (Mg^{2+} + K^{+})-

Table 3. Effect of various inhibitors on membrane-bound $(Ca^{2+} + Mg^{2+})$-ATPase activity. Oligomycin, DCCD and DES were added in 95% ethanol, making a final ethanol concentration of 0.95%

Inhibitor	Enzyme activity (μmol. Pi/hr/mg protein)		
	Ca^{2+} added		
	None	100 μM	Ca^{2+} stimulation (% control)
None (control)	358	591	100
Oligomycin			
2 μg/ml	343	584	103
5 μg/ml	355	597	104
10 μg/ml	357	605	106
DCCD			
10 μM	340	529	81
100 μM	356	494	59
DES			
10 μM	376	535	68
100 μM	400	516	50
0.95% ethanol	320	543	96
Na_2MoO_4			
0.5 mM	137	358	95
1.0 mM	133	343	90
NaN_3			
0.1 mM	374	556	78
0.5 mM	358	565	89
1.0 mM	389	601	91
Na_3VO_4			
1 μM	294	516	95
10 μM	225	426	86
100 μM	143	364	95

DCCD = dicyclohexylcarbodiimide; DES = diethylstilbestrol.

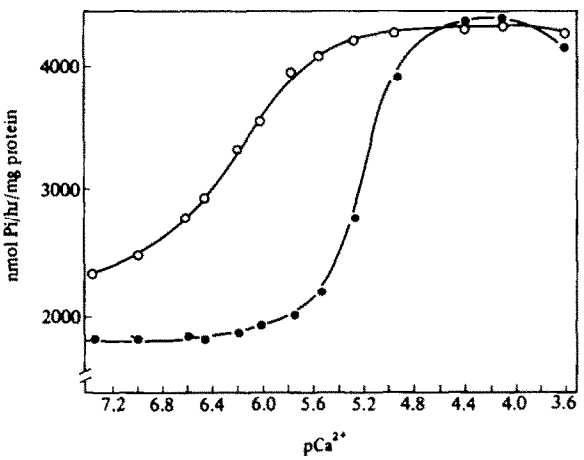

Fig. 3. Effect of increasing free Ca^{2+} concentration on membrane-bound $(Ca^{2+} + Mg^{2+})$-ATPase. The levels of free Ca^{2+} in the assay mixtures were buffered with EGTA (○) or CDTA (●), both at 200 μM. Each assay contained 22.7 μg of membrane protein. CDTA: *trans*-cyclohexane-1,2-diamine-*N,N,N'N'*-tetraacetic acid.

ATPase (data not shown). Buffer containing 2 M $NaClO_4$ eluted Ca^{2+} + ATPase with higher specific activity and very little $(Mg^{2+} + K^+)$-ATPase activity. This material was concentrated, dialysed and applied to a cellulose cation-exchange column (Fig. 5). The enzyme at this stage of purification lost activity rapidly at salt concentrations of less than 0.25 M, so these operations were carried out rapidly. Elution with a buffer containing 0.1 M NaCl removed a large amount of protein, including a considerable amount of ATPase that does not require Ca^{2+}. Buffer containing 1 M NaCl eluted the Ca^{2+}-ATPase in a sharp peak. This material was used for most of the subsequent characterization of the solubilized enzyme. The purification is summarized in Table 4.

Properties of solubilized, partially purified enzyme

Chromatography of the enzyme on a calibrated Sephadex G-100 column resulted in elution of the major peak of activity at a position corresponding to a M_r of $105 \pm 6 \times 10^3$. The substrate specificity (Table 5) was not greatly changed from that of the membrane preparation. The pH profile (Fig. 2) also was similar to that of the membrane-bound enzyme, except that the high pH shoulder was gone. It was noted during purification that the solubilized enzyme was unstable at low salt concentrations. The optimum salt concentration for assay was about 125 mM (Fig. 6), with suppression of Ca^{2+}-stimulation at higher concentrations, even though much higher concentrations were used to stabilize the enzyme during chromatography and storage. Addition of phosphatidylcholine or phosphatidylserine had no effect on activity (data not shown).

Enzyme carried through the purification process only as far as the hexyl-Agarose step was used to determine the Ca^{2+}-binding affinity. A Lineweaver–Burk plot of the data (Fig. 7) was biphasic, with apparent K_as of 0.06 and 15 mM Ca^{2+}. In these assays the enzyme, which had been eluted in buffer containing 2 M $NaClO_4$, was diluted to a salt concentration of 100 mM. In a similar experiment using enzyme eluted from CM-cellulose with 1 M NaCl (data not shown), enzyme diluted to 50 μM NaCl gave a single line with an apparent K_a of 0.5 μM; when 125 mM KCl was added, a biphasic curve essentially identical to Fig. 7 was obtained. The calmodulin inhibitors fluphenazine [8] and ophiobolin A [9] inhibit strongly (Fig. 8). Beef brain calmodulin (2.5 units), when added to an assay mixture containing 1 mM $CaCl_2$, stimulated the enzyme by about 30% (data not shown).

DISCUSSION

Detection of the enzyme described in this paper requires assay under conditions quite different from those usually used for assay of plant ATPases, i.e. low substrate concentration and no added Mg^{2+}, but with added Ca^{2+}. Both the membrane preparations from which the enzyme is prepared and the ATP used for assay ordinarily contain trace amounts of Mg^{2+}. Chelation of Mg^{2+} with CDTA eliminates activation by low levels of Ca^{2+}, suggesting that the enzyme is a $(Ca^{2+} + Mg^{2+})$-ATPase.

This enzyme most closely resembles a high-affinity $(Ca^{2+} + Mg^{2+})$-ATPase found in the plasma membranes of animal cells [10]. Both enzymes have broad specificity for nucleoside triphosphates, with little activity on pNPP.

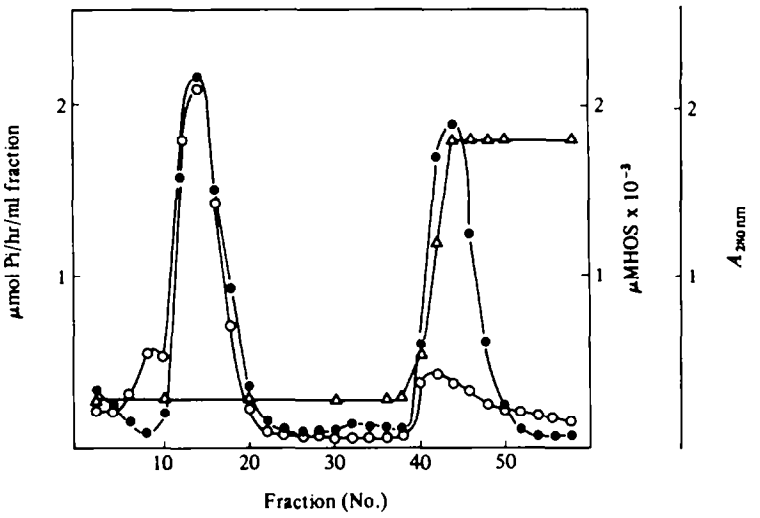

Fig. 4. Chromatography of detergent extract on hexyl-agarose. ●——●, $(Ca^{2+} + Mg^{2+})$-ATPase, ○——○, A 280 nm; Δ——Δ, conductivity, μMHO. The assays for $(Ca^{2+} + Mg^{2+})$-ATPase contained 10 mM Hepes-imidazole, pH 6.5, 100 μM $CaCl_2$, 100 mM $NaClO_4$ and 100 μM ATP. Fractions are 5 ml.

Table 4. Purification of $(Ca^{2+} + Mg^{2+})$-ATPase

Fraction	Activity (μmol. Pi/hr)	Recovery (%)	Specific activity (μmol. Pi/hr/mg protein)	Purification (fold)
Plasma membrane—enriched microsome fraction	9.2	100	2.3	1
High salt hexyl agarose ATPase	14.7	159	6.7	3
High salt CM-Cellulose ATPase	6.3	69	56.4	25

Table 5. Substrate specificity of partially purified $(Ca^{2+} + Mg^{2+})$-ATPase

Substrate	Enzyme activity (μmol. Pi/hr/mg protein)			
	200 μM EGTA	1 mM Ca^{2+}	Ca^{2+} stim.	% of ATP Ca^{2+} stim.
ATP	79.1	131.0	51.9	100
GTP	33.0	50.8	17.8	34
CTP	74.4	115.5	41.1	79
UTP	66.9	110.9	44.0	85
*p*NPP	28.3	29.4	1.1	2

Although such broad specificity has been suggested as evidence for phosphatase activity [2], neither the pH profile nor the effects of inhibitors on the enzyme described here are consistent with phosphatase activity. The animal enzyme is activated half-maximally at 13 μM free Ca^{2+}, with a Hill coefficient of 1.4. Mg^{2+} is required for activity but is not ordinarily added to assays because the requirement is met by endogenous Mg^{2+} in the enzyme preparations and substrates [10]. The divalent metal ion requirements of the plant enzyme are very similar to those of the animal enzyme. The animal enzyme requires a protein activator that is not calmodulin [10]

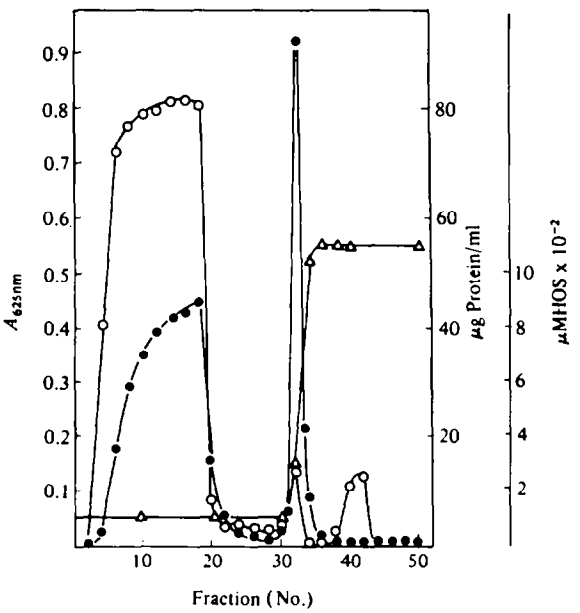

Fig. 5. Chromatography of $(Ca^{2+} + Mg^{2+})$-ATPase on CM-cellulose ion exchange column. ●——●, $(CA^{2+} + Mg^{2+})$-ATPase (A 625 nm); ○——○, protein; Δ——Δ, conductivity. Assay conditions are as in the legend to Fig. 4 except 100 mM NaCl replaces 100 mM $NaClO_4$. Fractions are 4.6 ml.

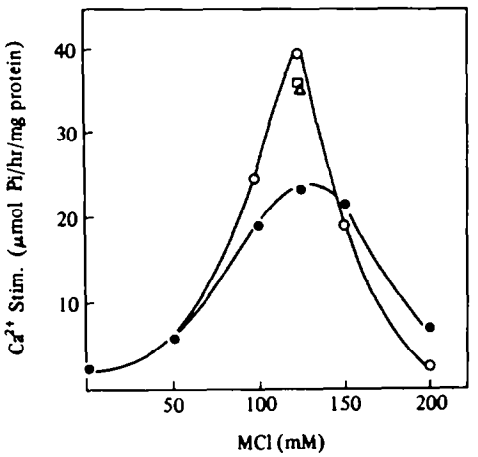

Fig. 6. Effect of varying concentrations of alkali metal and ammonium chlorides on activity of $(Ca^{2+} + Mg^{2+})$-ATPase partially purified by chromatography on CM-cellulose. The assay was as in the legend to Fig. 4 except the $NaClO_4$ was replaced by varying amounts of salt. ●——●, NaCl; ○——○, KCl; □——□, NH_4Cl; Δ——Δ, LiCl.

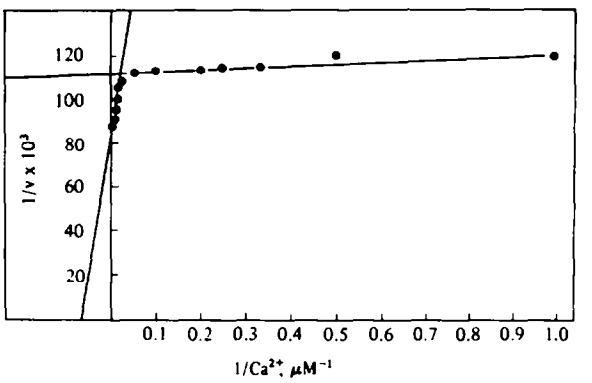

Fig. 7. Lineweaver–Burk plot of the effect of varying Ca^{2+} concentrations on the activity of $(Ca^{2+} + Mg^{2+})$-ATPase partially purified by chromatography on hexyl agarose. Assay conditions as in the legend to Fig. 4 except that the $CaCl_2$ concentration was varied.

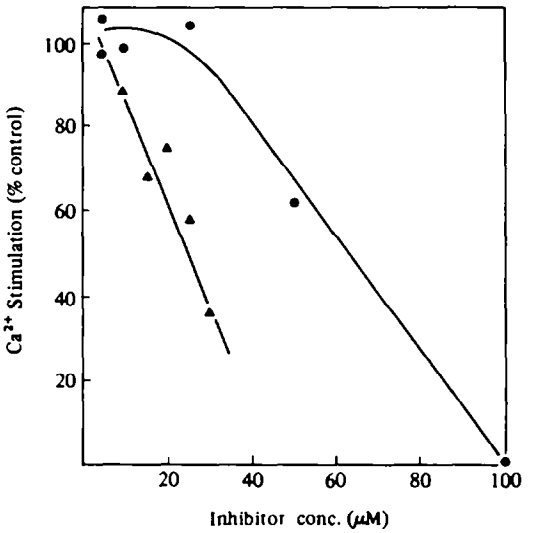

Fig. 8. Inhibition of $(Ca^{2+} + Mg^{2+})$-ATPase partially purified by chromatography on CM-cellulose by two calmodulin inhibitors. ●——●, Fluphenazine ▲——▲ ophioboilin A. Control activity was 33.7 μmol. Pi/hr/mg protein.

and is not affected by trifluoperazine, a typical calmodulin inhibitor. Although the plant enzyme responded to added calmodulin, the response is so weak as to be of doubtful significance. Two calmodulin inhibitors, fluphenazine and ophiobolin A, inhibit the solubilized and partially purified plant enzyme. Although one would expect endogenous calmodulin to have been separated from the enzyme during the purification process, it is possible that calmodulin is a tightly bound subunit of the enzyme, as it is of phosphorylase kinase [11]. Relatively high concentrations of inhibitor are required for substantial inhibition of phosphorylase kinase [12] as well as of the ATPase described here. Calmodulin inhibitors frequently inhibit other Ca^{2+}-binding proteins also [8], so it is not possible to decide, from inhibitor studies alone, whether calmodulin is involved with the $(Ca^{2+} + Mg^{2+})$-ATPase.

EXPERIMENTAL

Isolation of a plasma membrane—enriched microsomal fraction. Maize seedlings (B73Ht x Mo17Ht) were grown at 28° in the dark for three days. The etiolated shoots were removed from the seedlings with a razor blade, washed with deionized water and

weighed. The shoot segments were added to buffer (buffer A: 0.25 M sucrose, 20 mM imidazole, adjusted to pH 7.2 with HCl; 4 ml/g shoots), homogenized in a Waring blender, and the homogenate transferred to a Polytron homogenizer for 30 sec at setting '4' followed by 60 sec at setting '6'. Polyvinylpyrrolidone (0.25 g/g shoots) was added and thoroughly mixed. The homogenate was filtered through one layer of Miracloth (Calbiochem-Behring Corp., San Diego, CA) to remove the bulk of the fibrous material. The filtrate was centrifuged in a Beckman 45 TI rotor at 80000 *g* for 30 min. The microsomal pellet obtained was resuspended in buffer A and layered on a two-step sucrose-density gradient (34% over 45%) in 20 μM imidazole–HCl, pH 7.2. The gradient was centrifuged in a SW-27 swinging-bucket rotor in a Beckman L-8 centrifuge using the slow acceleration mode for 2 hr at 112840 *g*. The plasma membrane-enriched fraction from the microsome was collected at the 34%/45% sucrose interface.

Detergent extraction. The plasma membrane-enriched fraction was dialysed for 12 hr against buffer A at 4°, then extracted with 1% Zwittergent-3,12 (Calbiochem-Behring Corp.) in 1 M KCl. The undissolved membrane material was removed by centrifuging at 80000 *g* for 30 min. The supernate was dialysed for 6 hr against buffer A, followed by 12 hr against buffer B (0.25 M $NaClO_4$, 20 mM imidazole adjusted to pH 8.0 with HCl).

Chromatography of solubilized Ca^{2+}-ATPase. The dialysis retentate was applied to a 1.6 × 30 cm column of hexyl Agarose (Miles-Yeda Laboratories) previously equilibrated with buffer B. The column was eluted with 60 ml of buffer B followed by 60 ml of buffer C (2 M $NaClO_4$, 20 mM imidazole–HCl, pH 8.0). The column fractions (5 ml) were assayed for Ca^{2+}-ATPase and ($Mg^{2+} + K^+$)-ATPase, and the absorbance at 280 nm and the conductivity were measured.

The Ca^{2+}-ATPase eluted from the hexyl Agarose column with buffer C was dialysed against buffer D (1 M NaCl, 20 mM imidazole–HCl, pH 8.0) for 24 hr at 4°. The dialysis retentate was concentrated with Aquacide II-A (Calbiochem-Behring Corp.) and then, immediately before application to a 1.6 × 15 cm CM-cellulose column, diluted with deionized water to 0.1 M NaCl concentration. The enzyme soln was applied to the column, and the column was eluted with 60 ml 0.1 M NaCl, 10 mM imidazole–HCl, pH 8.0, followed by 60 ml 1 M NaCl, 20 mM imidazole–HCl, pH 8.0. The enzyme loses activity in salt solutions less concentrated than 0.25 M, so these steps must be carried out rapidly.

The Ca^{2+}-ATPase obtained by elution of the CM-cellulose column with 1 M NaCl buffer was concentrated with Aquacide II-A to approximately 10 ml and a portion was applied to a Sephadex G-100 column (2.6 × 86 cm) which was equilibrated with 1 M NaCl, 20 mM imidazole–HCl, pH 8.0. The column was eluted with the same buffer at a flow rate of 1 ml/min.

Enzyme assays. ($Mg^{2+} + K^+$)-ATPase was assayed in 33 mM MES adjusted to pH 6.0 with Tris base, 50 mM KCl, 1.5 mM $MgCl_2$, 3 mM ATP [13]. The assay mixture for Ca^{2+}-ATPase contained 10 mM Hepes adjusted to pH 6.5 with imidazole, with and without 100 μM $CaCl_2$, and 100 μM ATP. After solubilization of the enzyme, the assay mixtures contained, in addition, 125 mM KCl or NaCl. There were no chelating agents added to the assay mixtures unless stated. Inorganic phosphate released in the ATPase assays was estimated by using a malachite green procedure [14]. All data points represent the average of 2 or 3 replicate measurements.

Protein determinations were made by using a trichloroacetic acid precipitation variation of the Lowry procedure [15].

The levels of magnesium and calcium in certain assay mixtures were estimated by atomic absorption spectrophotometry with a Varian Techtron Model 1200 atomic absorption spectrometer with a combination $Ca^{2+}/Mg^{2+}/Ni^{2+}$ lamp source. Calculations of the free divalent metal ion concentration in the assay mixtures were performed by using equations from Pershadsingh and McDonald [5]. The following apparent association constants have been calculated for pH 6.5 by using the true association constants from ref. [5] (log K^1, M^{-1}): Ca^{2+}EGTA, 5.85; Ca^{2+}CDTA, 5.86; Ca^{2+}ATP, 3.41; MgEGTA, 0.06; Mg^{2+}CDTA, 5.33; Mg^{2+}ATP, 4.09.

Fluphenazine was a gift from the Smith Kline Beckman Corp. and ophiobolin A was prepared as described [9].

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