A CALMODULIN-DEPENDENT, MICROSOMAL ATPase FROM CORN (*ZEA MAYS* L.)

Peter DIETER and Dieter MARMÉ
Institut für Biologie III, University of Freiburg, Schänzlestrasse 1, 7800 Freiburg, FRG

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1. Introduction

In plants, calcium participates in the basic structural organization of the cell and plays an important role in regulating many biochemical and physiological processes. Some of them, e.g., the NAD kinase [1,2] and the microsomal Ca^{2+}-transport [3] are known to be mediated by calmodulin (CaM).

Evidence has been presented that the cytoplasmic free $[Ca^{2+}]$ in plants is regulated by an active accumulation of Ca^{2+} into the mitochondria and an active extrusion through the plasma membrane [4]. The Ca^{2+} transport in a plasma membrane-enriched microsomal fraction has been characterized in some detail [5]. We have shown that the Ca^{2+} transport into EDTA-washed plant microsomes can be stimulated by calmodulin [3]. Preliminary experiments have shown that light inhibits specifically the CaM-dependent part of the transport whereas the CaM-independent part is not changed (P. D., D. M., unpublished). The isolation and purification of the microsomal Ca^{2+}-transport ATPase is a prerequisite for the investigation of its biochemical and regulatory properties. Similar attempts have been made to isolate a Ca^{2+}-ATPase from the red blood cell plasma membrane [6]. However, in this case the CaM-dependence of the ATPase was lost after purification and could only be restored by adding phosphatidylcholine [7].

Here, we report on the solubilization and partial purification of a plant microsomal ATPase by CaM–Sepharose affinity chromatography and on the CaM-dependence of the partially purified enzyme.

2. Materials and methods

2.1. *Preparation of the CaM-dependent microsomal ATPase*

Corn (*Zea mays* L., Inracorn, category 5A, 3070, from Hambrecht, Freiburg) was grown on vermiculite at 25°C in total darkness for 5.5 days. About 100 g coleoptiles were harvested and homogenized as in [4] on ice in 200 ml buffer A (25 mM MOPS, titrated with 25 mM Tris to pH 7.5, 10% (w/w) sucrose and 5 mM EDTA). After centrifugation of the homogenate at 6000 × *g* for 15 min the pellet was discarded and the supernatant was recentrifuged at 48 000 × *g* for 20 min. The resulting pellet was resuspended in buffer B (buffer A without EDTA). This microsomal fraction (~5 mg protein/ml buffer) was solubilized with 1 mg Triton X-100/mg protein at 4°C for 10 min, then NaCl and $CaCl_2$ were added to final conc. 0.5 M and 5 mM, respectively. The unsolubilized material was pelleted at 100 000 × *g* for 30 min. The resulting supernatant containing the solubilized CaM-dependent ATPase was loaded onto a CaM–Sepharose affinity column which was equilibrated with buffer C (buffer B with 0.5 M NaCl, 5 mM $CaCl_2$ and 0.05% Triton X-100). About 100 mg bovine brain calmodulin (purified essentially according to [8]) was coupled to 5.5 g CNBr-activated Sepharose 4B as in [2]. After loading, the column was washed with 2–3 bed vol. buffer C. Proteins were eluted with buffer D (buffer C without $CaCl_2$ but with 10 mM EGTA). To the eluted fractions $CaCl_2$ was added to 15 mM final conc. All assays were made immediately after elution to avoid as much as possible the loss of enzyme activity. The whole procedure from the harvest to the enzyme assay took ~12 h.

2.2. *ATPase assay*

The incubation medium contained in 0.5 ml final vol. buffer B 5 mM $MgCl_2$, 1 mM $CaCl_2$, 10^{-6} M oligomycin and 1 mM ATP. Calmodulin (~550 nM) and 50 μM fluphenazine [9] were added as indicated. The reaction was started by adding 50 μl of the various enzyme preparations. The mixture was incubated at 37°C for 30 min. During this time the enzyme kinetics

Table 1
Solubilization of ATPase activity from the microsomal fraction

Fraction	Protein (mg)	ATPase activity					
		Total (munit)			Specific (munit/mg protein)		
		−CaM	+CaM	+CaM + flu[a]	−CaM	+CaM	+CaM + flu
Microsomal	2.40	117[b]	125 (8)[c]	119	49	52(3)	50
100 kS	1.60	81	91(10)	76	51	57(6)	48
100 kP	0.88	45	47 (2)	45	51	53(2)	51

[a]flu, fluphenazine; [b]Each assay contained 50 μg protein and 0.02% Triton X-100 [c]CaM-dependent ATPase activity in brackets is defined as the difference of the ATPase activities with and without CaM

were linear. The reaction was stopped by adding 0.5 ml cold 10% (w/v) trichloroacetic acid. The mixture was centrifuged at 20 000 × *g* for 10 min and the inorganic phosphate was determined as in [3]. One unit of ATPase is defined as 1 μmol ATP hydrolysed/min at 37°C. The reproducibility of the assay was within ± 5%.

Proteins were determined as in [3] except for calmodulin which was determined by weight.

3. Results

A microsomal fraction isolated from etiolated corn coleoptiles contains a CaM-dependent ATPase activity which can be solubilized with Triton X-100. Table 1 shows the result of such an experiment: The CaM-dependent ATPase activity in the microsomal fraction is only ~6% of the total ATPase activity. After solubilization and centrifugation, ~70% of the total protein and 70% of the total ATPase activity remain in the 100 000 × *g* supernatant (100 kS). The CaM-dependent ATPase activity in the 100 kS is ~12% of the total ATPase activity. That means that most of the CaM-dependent microsomal ATPase activity has been solubilized and that a 2-fold purification has been achieved. Fluphenazine, an antipsychotic drug which is known to inhibit CaM-dependent processes [9] also inhibits the CaM-dependent ATPase activity either in the membrane-bound or in the solubilized form.

The solubilized fraction (100 kS) was applied onto a CaM–Sepharose column. All material coming through the column including the salt wash was pooled (wash fraction). Proteins which were bound to the CaM–Sepharose in the presence of calcium were eluted by EGTA (EGTA fraction). As shown in table 2, ~90% of the total protein and almost 99% of the total ATPase activity appear in the wash fraction. CaM-dependence of the ATPase activity in this fraction could no more be detected. Less than 2% of the total protein and ~2% of the total ATPase activity remained on the column and could be eluted by adding EGTA. This ATPase activity can now be stimulated by calmodulin

Table 2
Chromatography of the solubilized microsomal ATPase on a CaM–Sepharose affinity column

Fraction	Protein (mg)	ATPase activity					
		Total (munit)			Specific (munit/mg protein)		
		−CaM	+CaM	+CaM + flu[a]	−CaM	+CaM	+CaM + flu
100 kS	22.8	689[b]	745(56)[c]	588	30	33 (3)	26
Wash	20.3	683	676 (−)	680	34	33 (−)	33
EGTA	0.34	15[d]	35(20)	11	44	103(59)	32

[a]flu, fluphenazine; [b]Each assay of the 100 kS and the wash fraction contained 50 μg protein and 0.05% Triton X-100 [c]CaM-dependent ATPase activity in brackets is defined as the difference of the ATPase activities with and without CaM [d]Each assay of the EGTA fraction contained 2 μg protein and 0.05% Triton X-100

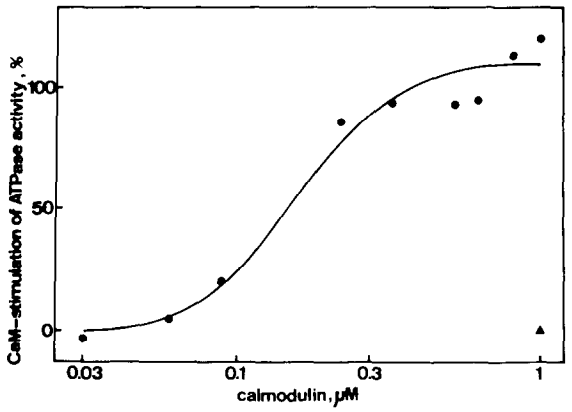

Fig.1. Dose–response curve of the activation of partially purified microsomal ATPase from corn by bovine brain calmodulin. All assays were carried out with 2 μg EGTA eluate proteins and 0.05% Triton X-100. Fluphenazine (50 μM) was added at saturating amounts of calmodulin (▲). The specific ATPase activity in the absence of calmodulin was 48 munit/mg protein.

by >130%. Fluphenazine abolishes completely the CaM-dependent activation. Fig.1 shows the ATPase activity of the EGTA fraction as a function of the amount of added calmodulin. At saturating amounts of calmodulin the ATPase activity is enhanced ~2-fold. Half-maximal stimulation is achieved at ~0.16 μM calmodulin. Fluphenazine, added to the assay mixture, inhibits completely the stimulation by calmodulin (fig.1, ▲).

4. Discussion

ATP-dependent Ca^{2+} uptake into a microsomal fraction from higher plants [5] has been reported to be enhanced by calmodulin [3]. As shown in table 1, such a microsomal fraction contains a CaM-dependent ATPase activity. This activity (3 munit/mg protein) is very small as compared to the 49 munit/mg protein of the CaM-independent ATP-hydrolysing activity. However, assuming a stoichiometry of 1 mol Ca^{2+} transported/mol ATP hydrolysed, one can calculate from the CaM-dependent transport activity [3] the CaM-dependent specific ATPase activity to be ~1 munit/mg protein. Taking into account that only the inside-out vesicles derived from the plasma membrane (for discussion see [4]) contribute to the Ca^{2+} transport and that not all vesicles are sealed, this calculated minimal value is not too different from the observed 3 munit/mg protein. But it cannot completely be excluded that the microsomal fraction contains another CaM-dependent ATPase which is not involved in Ca^{2+}-transport.

After solubilization of the microsomal fraction with Triton X-100 the CaM-dependent ATPase activity is retained in the presence of Ca^{2+} on the CaM–Sepharose column (table 2). No such activity could be detected in the wash fraction. The EGTA eluate contains only ~2% of the CaM-independent ATP-hydrolysing activity but 36% of the CaM-dependent ATPase activity. This CaM-dependent ATPase activity is quite unstable; at 4°C it disappears within 24 h. This is probably the reason for the low recovery of the CaM-dependent ATPase activity from the affinity column. A 20-fold purification of the CaM-dependent ATPase activity can be calculated from the specific activities of the 100 kS and the EGTA fraction.

The specific activity of the CaM-independent ATPase is also increased from 30 munit/mg protein in the 100 kS to 44 munit/mg protein in the EGTA fraction (table 2). These data can be interpreted at least in two ways:

(1) An ATPase has been copurified which binds in a Ca^{2+}-dependent manner to calmodulin but can no more be activated by calmodulin after elution. Such an ATPase has been isolated from red blood cells by CaM–Sepharose affinity chromatography [6]. CaM-sensitivity of this ATPase can only be restored by adding phosphatidylcholine [7].

(2) The solubilized CaM-dependent ATPase from plants has a basal CaM-independent activity. This is supported by the fact that EDTA-washed plant microsomes possess a Ca^{2+}-transport activity which cannot be inhibited by fluphenazine [3] and thus does not depend on calmodulin.

The biochemical properties of the plant microsomal Ca^{2+}-transport ATPase will be further investigated with special emphasis on the regulation of the ATPase activity. Preliminary experiments have shown that light inhibits specifically the CaM-dependent part of the plant microsomal Ca^{2+}-transport whereas the CaM-independent part is not changed (P. D., D. M., unpublished). Investigation of the mechanism of these light-mediated changes at the enzyme level will be of special interest because alterations of the microsomal Ca^{2+}-transport activity could cause variations of the free cytoplasmic Ca^{2+}-level and therefore of Ca^{2+}-dependent physiological processes in higher plant cells.

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References

[1] Anderson, J. M., Charbonneau, H., Jones, H. P., McCann, R. O. and Cormier, M. J. (1980) Biochemistry 19, 3113–3120.

[2] Dieter, P. and Marmé, D. (1980) Cell Calcium 1, 279–286.

[3] Dieter, P. and Marmé, D. (1981) Proc. Natl. Acad. Sci. USA, in press.

[4] Dieter, P. and Marmé, D. (1980) Planta 150, 1–8.

[5] Gross, J. and Marmé, D. (1978) Proc. Natl. Acad. Sci. USA 75, 1232–1236.

[6] Niggli, V. Penniston, J. T. and Carafoli, E. (1979) J. Biol. Chem. 254, 9955–9958.

[7] Penniston, J. T., Graf, E., Niggli, V., Verma, A. K. and Carafoli, E. (1980) in: Calcium Binding Proteins and Calcium Function (Siegel, F. L. et al. eds) vol. 14, pp. 23–30, Elsevier/North Holland, Amsterdam, New York.

[8] Jamieson G. A. jr and Vanaman, T. C. (1979) Biochem. Biophys. Res. Commun. 90, 1048–1056.

[9] Wolff, D. J. and Brostrom, C. O. (1976) Arch. Biochem. Biophys. 173, 720–731.