RESEARCH COMMUNICATION

Identification and characterization of ATPase activity associated with maize (*Zea mays*) annexins

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An ATPase activity is associated with maize (*Zea mays*) annexins. It has a pH optimum of 6.0, shows Michaelis–Menten kinetics and is not stimulated by Ca^{2+}, Mg^{2+}, EDTA or KCl; it is not inhibited by vanadate, molybdate, nitrate or azide, but *N*-ethylmaleimide inhibits by ~ 30% at 1–2 mM. These properties indicate that the activity is unlike other ATPases, although it has many features in common with the myosin ATPase. Gel filtration shows that the ATPase activity is mainly associated with a 68 kDa protein that is extracted with the p33/p35 annexins and cross-reacts with antibodies to these proteins.

INTRODUCTION

Annexins are Ca^{2+}-dependent phospholipid-binding proteins with sequence similarity over four or eight repeats, each of about 70 amino acids. A wide range of functions has been suggested for annexins in animals, including inhibition of phospholipase A_2, regulation of membrane–cytoskeleton interactions, control of exocytosis and endocytosis, and inositol cyclic phosphate phosphohydrolase activity (see Creutz, 1992; Moss, 1992; Gruenberg and Emans, 1993; Raynal and Pollard, 1994). In plants, annexin-like proteins have been identified on the basis of antibody cross-reactivity, Ca^{2+}-dependent membrane binding, and protein sequence data (Boustead et al., 1989; Smallwood et al., 1990; Blackbourn et al., 1991, 1992; Clark et al., 1992; Andrawis et al., 1993). However, very little is known of the functions of plant annexins, although there is evidence for a role in exocytosis [reviewed by Battey and Blackbourn (1993)], and in enzyme regulation (Andrawis et al., 1993). We report here that maize (*Zea mays*) annexins have ATPase activity; its characteristics are described and the implications for our understanding of plant annexin function are briefly discussed.

MATERIALS AND METHODS

Materials

Maize (*Zea mays* L., cv. Clipper) seed was soaked overnight in running water and then grown for 5 days in moist vermiculite in the dark at approx. 22 °C. The etiolated coleoptiles and enclosed leaf rolls were harvested, frozen in liquid N_2 and stored at −18 °C until required.

Isolation and purification of annexins from maize

Annexins were isolated from maize coleoptiles as described previously (Blackbourn et al., 1991), except that following hydroxyapatite chromatography the protein, which was eluted in 500 mM K_2PO_4/KH_2PO_4, was dialysed overnight against 4 × 2 litre changes of 10 mM Tris/HCl, pH 7.3, to remove all traces of phosphate and concentrated using Amicon Centricon-10 micro-concentrators (Stonehouse, Glos., U.K.) according to the manufacturer's instructions. Annexins were further purified by gel filtration on Superdex 75 (Pharmacia LKB): a post-hydroxyapatite annexin preparation was chromatographed in 10 mM Tris/HCl (pH 7.3)/0.1 M NaCl/1 mM $MgCl_2$, and 0.5 ml fractions were collected. Protein and ATPase activity were measured and a sample of protein precipitated by the addition of trichloroacetic acid to a final concentration of 10% (w/v). After 3 h on ice, the precipitated protein was pelleted by centrifugation at 16000 *g* for 10 min and resolved by SDS/PAGE.

Enzyme assays

P_i-liberating activity was measured in a 0.5 ml reaction volume containing 3 μg of annexins, 3 mM substrate and 30 mM Tris/Mes at the desired pH. When required, $MgSO_4$ was added to a final concentration of 3 mM. Potential inhibitors were added before the addition of annexins and the substrate. The reaction was started by the addition of substrate. Following incubation at 37 °C for 15 min, the reaction was terminated by the addition of 1.5 ml of ice-cold Ames (1966) Reagent. Colour development was allowed to occur for 45 min at room temperature, after which time the A_{820} was measured against reagent blanks. Calibration curves were constructed in the range 0–120 nmol of P_i using reaction conditions identical with those employed for the assays. As some acid hydrolysis of nucleotides occurred in the absence of annexins, controls of nucleotide only were included and activity calculated as the difference in activity in the presence and absence of annexins. At least three replicates were carried out for each assay, and results are expressed as means ±S.E.M. In order to remove any tightly bound Mg^{2+}, a post-hydroxyapatite annexin fraction was passed through a column of cation-chelating resin (Sigma C 7901; volume 0.2 ml) and 3 μg annexin was assayed for ATPase activity in the presence or absence of 3 mM Mg^{2+}.

Protein assay and electrophoresis

Protein concentrations were measured colorimetrically with BSA as the standard (Sedmak and Grossberg, 1977). Analysis by SDS/PAGE was performed on 12% gels as described by Laemmli (1970), with molecular-mass standards from Sigma (Dalton mark VII). Gels were stained with Coomassie Brilliant Blue.

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RESULTS

After hydroxyapatite chromatography the maize annexin preparation typically contains ~ 70% p33/p35; both of these proteins have sequence similarity to animal annexins [see Blackbourn et al. (1992)]. The other major proteins are p68, which cross-reacts with antibodies to p33/p35, and p23, which appears not to be an annexin on the basis of a lack of antibody cross-reactivity or sequence identity (Blackbourn et al., 1991, 1992). ATPase activity associated with this fraction ranges from 16 to 43 μmol of P_i/h per mg, according to the preparation. Activity is reduced by approx. 80% by boiling, and is linear for 15 min at 37 °C and 40 min at room temperature (McClung, 1994). The pH optimum is 6.0 (Figure 1), and the ATPase exhibits Michaelis-Menten kinetics over the range 0–3 mM ATP, with a K_m of 0.55 mM and $V_{max.}$ of 48 μmol of P_i/h per mg (Figure 2).

There is no requirement for Mg^{2+} for ATPase activity; inclusion of Mg^{2+} at the same concentration as ATP slightly reduces activity, whilst EDTA has no effect (Table 1). Passage over cation-chelating resin to remove tightly bound Mg^{2+} increases the ATPase activity slightly (Table 1). KCl stimulates some ATPases (Mommaerts and Green, 1954; Hodges et al., 1972), but there is no effect of 50 mM KCl on annexin ATPase, and activity is inhibited by about 30% by 500 mM KCl (Table 2). Plant annexins bind to phospholipids in the presence of Ca^{2+}, and this is probably functionally important (Blackbourn et al., 1991); however, annexin ATPase activity is unaffected by Ca^{2+} or EGTA at 1 mM (Table 2).

ATPases can often be distinguished from each other by their sensitivity to inhibitors. The annexin-associated ATPase is not inhibited by sodium vanadate, even at concentrations sufficient to cause almost complete inhibition of plant plasma-membrane ATPases (Gallagher and Leonard, 1982) (Table 3). The activity is also insensitive to sodium azide, a potent inhibitor of mitochondrial F_1-ATPase (Bowman et al., 1978), sodium molybdate, an acid phosphatase inhibitor (Leigh and Walker, 1980) and potassium nitrate, a tonoplast ATPase inhibitor (O'Neill et al., 1983) (Table 3). *N*-Ethylmaleimide, an inhibitor of the ATPase activity of myosin (Sekine et al., 1962) and *N*-ethylmaleimide-sensitive factor (Block et al., 1988; Tagaya et al., 1993) reduces activity of the annexin ATPase by ~ 30% at 1–2 mM (Table 3). ATP and GTP are hydrolysed equally effectively (Table 4). The next best substrates are CTP, ITP and UTP; ADP and AMP are poor substrates, and the acid phosphatase substrate *P*-nitrophenyl phosphate is not hydrolysed. Mg^{2+} is not needed for hydrolysis of any of these substrates, and, as with ATP, is typically slightly inhibitory (McClung, 1994).

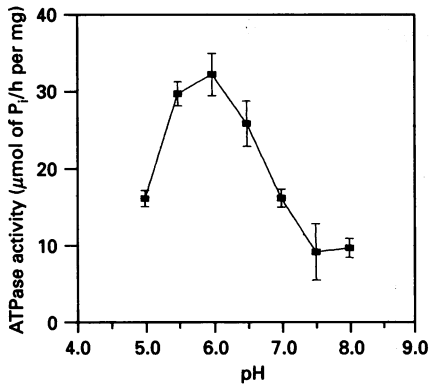

Figure 1 pH optimum of annexin ATPase activity

Assays were performed using 30 mM Tris/Mes over a range of pH values, with 3 μg of annexin, 3 mM $MgSO_4$ and 3 mM ATP.

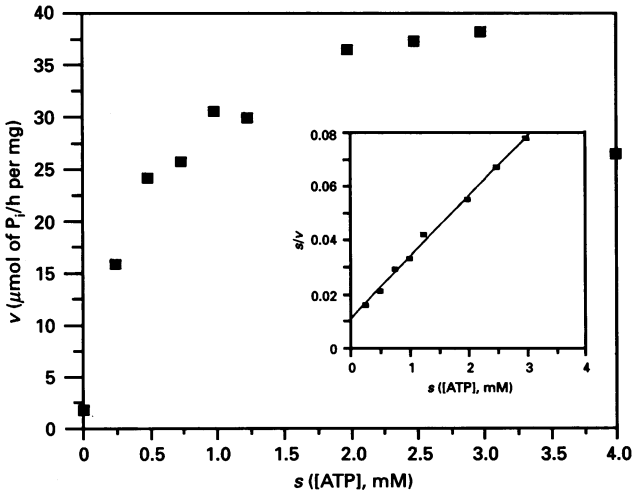

Figure 2 The effect of substrate concentration on annexin ATPase activity

Assays were performed at pH 6.0, with 1 μg of annexin and 3 mM $MgSO_4$. The inset shows a Hanes plot [substrate concentration (*s*)/velocity (*v*) against *s*]. The activity at 4 mM ATP was omitted.

Table 1 Effect of Mg^{2+} on annexin ATPase activity

ATPase assays containing 3 μg of annexins were carried out in the presence or absence of $MgSO_4$, the presence of 10 mM EDTA and after annexins were passed through a column containing a cation-chelating resin. Reactions were started by the addition of 3 mM ATP.

Treatment	ATPase activity (μmol of P_i/h per mg)	(% of control)
Control (−Mg^{2+})	32.9±2.6	–
+3 mM Mg^{2+}	27.2±2.6	82
+10 mM EDTA	33.0±2.0	100
Column eluate		
−Mg^{2+}	44.0±1.2	134
+3 mM Mg^{2+}	22.0±2.4	69
Annexin blank (no ATP)	4.5±1.0	5

Table 2 Effect of K^+ and Ca^{2+} on annexin ATPase activity

K^+ and Ca^{2+} were supplied as the chloride salts.

Treatment	ATPase activity (μmol of P_i/h per mg)	(% of control)
Control (−K^+)	25.3±2.1	–
+50 mM K^+	30.2±0.9	118
+500 mM K^+	18.1±0.8	71
Control (−Ca^{2+})	41.5±2.0	–
+1 mM EGTA	45.4±2.0	108
+1 mM Ca^{2+}	45.1±2.8	109

Table 3 Effect of various ATPase inhibitors on annexin ATPase activity

Inhibitors were added at the indicated concentrations to the standard reaction mixture before the addition of ATP. Assays containing sodium orthovanadate were carried out in the presence of 50 mM KCl. *N*-Ethylmaleimide was prepared fresh immediately before use. It was dissolved in Tris/Mes, pH 6.0, added to 5 μg of annexins at the indicated concentrations and left for 15 min on ice. The *N*-ethylmaleimide reaction was stopped by dilution into the standard ATPase reaction mixture, containing 0.2% dithiothreitol, before the addition of ATP. Activities were measured in the absence of $MgSO_4$.

Reagent	Concn. (mM)	ATPase activity (μmol of P_i/h per mg)	(% of control)
Sodium vanadate	(Control)	39.2 ± 1.8	–
	0.025	37.5 ± 2.1	96
	0.05	39.2 ± 1.0	100
	0.1	38.8 ± 1.4	99
N-Ethylmaleimide	(Control)	21.7 ± 3.9	–
	1	13.7 ± 2.1	63
	2	16.5 ± 1.6	76
Sodium molybdate	(Control)	43.2 ± 0.85	–
	0.1	39.4 ± 3.3	91
	1.0	41.2 ± 1.1	95
Sodium azide	0.1	47.4 ± 2.4	110
	1.0	43.4 ± 3.6	100
Potassium nitrate	50	38.7 ± 2.4	90

Table 4 Substrate specificity for annexin ATPase

All assays contained 3 μg of annexins and 3 mM substrate. Activities were measured in the absence of $MgSO_4$.

Substrate	ATPase activity (μmol of P_i/h per mg)	(% of rate with ATP)
ATP	20.1 ± 3.4	–
AMP	4.0 ± 1.8	20
ADP	9.6 ± 0.8	48
CTP	14.3 ± 3.1	71
GTP	22.2 ± 3.2	110
ITP	16.0 ± 3.2	80
UTP	12.9 ± 3.5	64
p-Nitrophenol phosphate	0.64 ± 1.5	3
Annexin only	0.9 ± 0.5	4

On gel-filtration chromatography peak ATPase activity is coincident with fractions enriched in p68 on SDS/PAGE; lower activity is associated with annexins p33 and p35 (Figure 3).

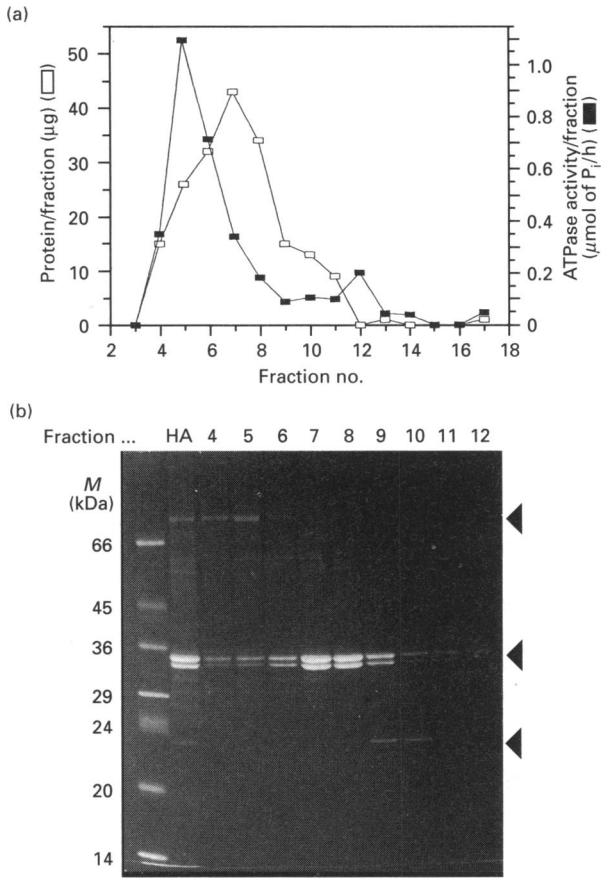

Figure 3 Gel-filtration chromatography and SDS/PAGE of ATPase and maize annexins

(**a**) Gel filtration. A Superdex 75 gel-filtration column (30 cm × 1 cm) was run in 10 mM Tris/HCl (pH 7.3)/0.1 M NaCl/1 mM $MgCl_2$. V_0 was 7.35 ml (fraction 4). (**b**) SDS/PAGE of fractions from gel filtration. p68, p35/p33 and p23 are indicated by ◀. Abbreviation: *M*, molecular mass.

DISCUSSION

This is the first report that annexins have ATPase activity. The post-hydroxyapatite fraction used for the characterization of this activity contains three annexins, p33, p35 and p68, that together constitute approx. 75% of the protein in that fraction. Gel filtration indicates that p68 has greatest ATPase activity; the specific activity of 170 μmol of P_i/h per mg places it at the higher end of the range of ATPase activities previously reported, exceeded only by some of the membrane transport ATPases [see, e.g., Dufour et al. (1988)]. These facts together allow us to be confident that the ATPase activity is not due to a co-purifying contaminant. However, the exact relationship between p68 and p33/p35 is currently unclear. Antiserum to maize annexins p33/p35 cross-reacts with p68 (Blackbourn et al., 1991); p68 may therefore be a covalent dimer of p33/p35. If this is the case, then dimerization appears to enhance ATPase activity. Annexin I is known to form stable dimers mediated by transglutaminase (Ando et al., 1989, 1991). Alternatively, p68 may be the monomeric native form of p33/p35 and the plant homologue of annexin VI. Antiserum to p33/p35 cross-reacts with chicken annexin VI (Blackbourn et al., 1991), but partial protein sequencing suggests greatest identity with annexins I, II, III and IV (Blackbourn et al., 1992). It is notable that annexin VI can bind ATP (Burgoyne and Geisow, 1989), and displays enhanced membrane binding in the presence of ATP (Geisow and Burgoyne, 1982). Lin et al. (1992) reported that annexin VI and ATP are essential for the budding of clathrin-coated pits during endocytosis. Furthermore, significant sequence similarity has been observed between the nucleotide-binding fold of the cystic-fibrosis transmembrane conductance regulator protein and the first repeat region in mammalian annexins (Chap et al., 1991). A final possibility is that p68 is distinct from p33/p35, but shares a common epitope.

The lack of requirement for Mg^{2+} for ATPase activity is unusual, but is also characteristic of myosin ATPase. Purified myosin ATPase is in fact strongly inhibited by the presence of Mg^{2+} (Hasselbach, 1957; Mühlrad et al., 1964) and, unlike the annexin ATPase, myosin ATPase is activated maximally by EDTA (Friess, 1954) and by 0.25–0.5 M KCl (Bowen and Kerwin, 1954; Mommaerts and Green, 1954) and can be activated by Ca^{2+} in the absence of K^+ (Seidal, 1959). The EDTA-stimulated myosin ATPase activity has no known physiological significance, since ATP is present as a complex with Mg^{2+} in the cell (Pollard et al., 1991). However, when myosin binds to actin to form actomyosin, the ATPase activity has a definite requirement for Mg-ATP. In the same way, annexin ATPase may only be fully active *in vivo* when bound to an as-yet-unidentified factor.

Our observation of ATPase activity in plant annexins is likely to have important implications for their function. A role in exocytosis, initially demonstrated for mammalian annexins (Ali et al., 1989), has been suggested in plants (Battey and Blackbourn, 1993), and the ability to hydrolyse nucleotide will be relevant to this. However, other intriguing possibilities, such as membrane–membrane motor activity, present themselves as exciting areas for future research.

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