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THE CALMODULIN-STIMULATED ATPase OF MAIZE COLEOPTILES FORMS A PHOSPHORYLATED INTERMEDIATE

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SUMMARY The calmodulin-stimulated ATPase of maize coleoptiles is a 140,000 M_r polypeptide. In the present study, formation of a phosphorylated intermediate by the enzyme is demonstrated. Phosphorylation is sensitive to chasing with unlabelled ATP and to hydroxylamine; lanthanum enhances its intensity while calmodulin enhances phosphorylation in the presence of lanthanum but not in its absence. Ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) inhibits phosphorylation of the purified enzyme, but microsome preparations give a band of phosphorylation of 153,000 M_r in its presence. This latter phosphorylated band was not abolished after a variety of permeabilising treatments in the presence of Triton X-100; phosphorylation of the enzyme was absent when sodium deoxycholate was used as the solubilising detergent. The identity of the 153,000 M_r band is discussed.

Maize (Zea mays L.) coleoptile plasma membrane contains a calmodulin-stimulated calcium-pumping ATPase (1,2), which catalyzes an nH^+/Ca^{2+} exchange (3). This enzyme has previously been shown (4) to be a polypeptide which shows common antigenicity with the erythrocyte membrane calcium pump, and has a similar M_r (140,000). The higher plant enzyme is believed to be the major transporter responsible for the maintenance of low cytoplasmic free calcium concentrations in higher plant cells (5). It is activated in the presence of increased calcium concentrations by the calcium-dependent regulator protein calmodulin (1).

In order to investigate the properties of the maize calmodulin-stimulated ATPase further, we have studied both the formation of phosphorylated intermediates by the purified enzyme and by microsomes containing it. Phosphorylated intermediate formation is a characteristic of ion-pumping

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ATPases of the E_1-E_2 type. The intermediate is an aspartyl phosphate which is sensitive to treatment with hydroxylamine and is sensitive to basic conditions. Turnover of the intermediate is rapid (6), but is stabilised by the presence of lanthanum (7) and acidic conditions such that it may readily be demonstrated using ATP labelled in the γ position with ^{32}P followed by precipitation of the protein in acidic conditions, separation of the proteins using polyacrylamide gel electrophoresis at pH 6 and autoradiography.

MATERIAL AND METHODS

Tissue and enzyme purification:
Enzyme and microsomes were extracted from 4.5d dark-grown maize (cv. Golden Bantam) seedlings by the methods described previously (4). The proteins in the "washed" microsome pellet were solubilised with Triton X-100 (0.1mg/mg protein or 1mg/mg protein), or sodium deoxycholate (1mg/mg protein) as indicated in the text. Where appropiate, the microsomes were permeabilised by being subjected to freeze/thaw cycles, vortexed for 10 min, or sonicated for 3 min in bursts of 15 s with 15 s cooling intervals (Dawe soniprobe, fine tip).

Phosphorylated intermediate formation:
The formation of phosphorylated intermediates in the microsome fraction and in purified enzyme was carried out by the method used previously for the erythrocyte calcium pumping ATPase (8). To the assays, microsome sample (100-200µg protein) or purified enzyme (2-10µg protein) were added. The reaction was started by the addition of either 37KBq/assay [γ-^{32}P]-ATP (New England Nuclear, 111MBq/mmol) or 370KBq/assay [γ-^{32}P]-ATP plus 2µM unlabelled ATP (Sigma, vanadate free) as indicated in text. The assay was conducted in Eppendorf tubes, which were incubated for 15 s on ice before reactions were terminated by addition of trichloroacetic acid (TCA) to 5% (w/v) final concentration. Calcium chloride (50µM final concentration), ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA, 500µM final concentration), lanthanum chloride (50µM final concentration) and calmodulin (Sigma, bovine brain, 25,000 units/0.53mg solid) were included as indicated in text. Where appropriate, 50µl 50mM unlabelled ATP was added for 1 min before addition of TCA. Hydroxylamine treatment was accomplished by washing the precipitated protein in 50mM hydroxylamine in 100mM 2(N-morpholino)ethanesulphonic acid (MES) brought to pH 6.0 with KOH. Control treatment (washing in MES/KOH) was also carried out and did not diminish phosphorylation. The pelleted protein was washed once in water and resuspended for electrophoresis in sodium phosphate sample buffer (pH 6.0) and incubated on ice for 30 min prior to electrophoresis on 5% or 7.5% gels prepared according to the method of Weber and Osborne (9) modified to pH 6.0. Gels were dried without fixation using a Biorad 483 slab gel drier and exposed for 1 d (microsome preparations) or 14 d (purified enzyme) at -70°C with Kodak X-omat S autoradiography film in the presence of a Du Pont Cronex Lightning-Plus intensifier screen. Gels were stained using Coomassie blue as described in (4) or by the method of (10). Values for M_r were determined by comparison of autoradiographs with M_r standards (Sigma SDS-6H) run in adjacent gel lanes which were cut out and stained as described in (4). Values given are representative of five experiments.

RESULTS AND DISCUSSION

Phosphorylation in presence of calcium:

In the presence of calcium, microsomes and purified enzyme showed a 140,000 M_r band of phosphorylation after 15 s on ice both in the presence of 37KBq

[γ^{32}P]-ATP per assay (fig.1, lane 1; fig.2, lane 2) and in the presence of 370KBq [γ-^{32}P]-ATP per assay plus 2μM unlabelled ATP (data not shown). In both cases, the 140,000 M_r polypeptide was not phosphorylated after chasing with unlabelled ATP (fig.1, lane 2; fig.2, lane 6) or after washing with hydroxylamine (fig.1, lane 3; fig.2, lane 7), indicating that it was an acyl-phosphate of the type described for ion-translocating ATPases of the E_1-E_2 type. This finding is identical to that of Niggli et al. (8) for the erythrocyte calcium-pumping ATPase and is within ±5,000 of the M_r established for the phosphorylated intermediate of that enzyme (11).
Two other bands of phosphorylation were also present in the microsome extract, one at 44,000 M_r and one at 28,000 M_r. The former (absent from the purified enzyme preparation) was not diminished by hydroxylamine and may, therefore be assumed to be the result of protein kinase activity. The latter was abolished by hydroxylamine and may be the result of partial proteolysis of the enzyme after phosphorylation.

Effect of calmodulin and lanthanum:
When microsome extracts and purified enzyme were incubated with lanthanum, apparent phosphorylation was increased as compared with that in the presence of calcium (fig. 3, lanes 2 and 4; fig. 2, lanes 2 and 4). Lanthanum reduces turnover of phosphorylated intermediates and thereby stabilises the phosphorylated state, resulting in apparent enhancement of phosphorylation (6). No reproducible effect of calmodulin (25 units/assay) on phosphorylation in the absence of lanthanum could be detected either in the case of microsomes or purified enzyme (fig. 3, lanes 1 and 2; fig. 2, lanes 1 and 2, apparent increased band intensity in lane 1, fig. 2 being the result of slightly heavier loading of that lane and not being found in all replicate experiments). However, in the presence of lanthanum, slight band intensification in the presence of calmodulin was suggested, at least for purified enzyme (fig. 2, lanes 3 and 4). This intensification could be demonstrated reproducibly. These effects of calmodulin are likely to indicate that increased enzyme activity in the presence of calmodulin is the result of increased turnover of the phosphorylated state rather than an increase in total phosphorylation at any instant. In the presence of lanthanum, inhibition of dephosphorylation results in the increased phosphorylation in the presence of calmodulin being detected as an intensified band.

Effect of EGTA:
When microsomes solubilsed with 0.1mg Triton X-100/mg protein were incubated in the presence of 500μM EGTA, a band of phosphorylation at M_r 153,000 was apparent while that at M_r 140,000 disappeared (fig. 4, lanes 1 and 2). This

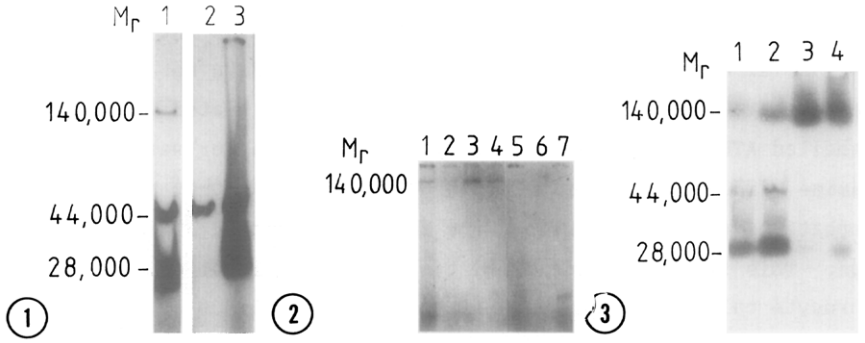

Figure 1. Phosphorylated intermediate formation by microsome fractions in the presence of calcium; autoradiograph of 5% pH 6.0 SDS polyacrylamide gel. Lane 1: membrane phosphorylated on ice for 15 s in the presence of 50μM added $CaCl_2$: lane 2: as lane 1 but 5mM unlabelled ATP added for 1 min after phosphorylation; lane 3: as lane 1 but pelleted membrane preparation washed with 50mM hydroxylamine in 100mM MES/KOH pH 6.0 prior to electrophoresis.

Figure 2. Phosphorylated intermediate formation by purified enzyme; autoradiograph of 7.5% pH 6.0 SDS polyacrylamide gel. Lane 1: enzyme incubated for 15 s on ice in the presence of 50μM added $CaCl_2$ and 25 units calmodulin; lane 2: as 1 but without calmodulin; lane 3: as 1 with 50μM $LaCl_3$ plus calmodulin; lane 4: as 3 without calmodulin; lane 5: as 1 but with 500μM EGTA and without calmodulin; lane 6: as lane 1 but 5mM unlabelled ATP added for 1 min after phosphorylation; lane 7: as lane 1 but pelleted protein washed with 50mM hydroxylamine in 100mM MES/KOH pH 6.0 prior to electrophoresis.

Figure 3. Effect of lanthanum and calmodulin on phosphorylated intermediate formation by microsomes. Autoradiograph of 5% pH 6.0 SDS polyacrylamide gel. Lane 1: Microsomes phosphorylated for 15 s on ice in the presence of 50μM added calcium plus 25 units calmodulin; lane 2: as 1, without calmodulin; lane 3: as lane 1 but with 50μM $LaCl_3$ plus calmodulin; lane 4: as 3, without calmodulin.

band was abolished by an ATP chase (fig. 4, lane 3), and was hydroxylamine sensitive (fig. 4, lane 4). To investigate whether persistence of phosphorylation in the presence of EGTA was due to the microsomes being insufficiently permeabilised to permit the chelation of calcium in the immediate environment of the enzyme by EGTA, a series of permeabilisation and solubilisation treatments were carried out. Tip sonicating or vortexing the microsomes solubilised with 0.1mg Triton X-100/mg protein did not diminish the phosphorylation of the 153,000 M_r polypeptide in the presence of EGTA (Fig. 5a, lanes 1-4). Freeze/thawing the microsomes solubilised with 0.1mg Triton X-100/mg protein two, three or four times did not diminish the phosphorylation at 153,000 M_r in the presence of EGTA (Fig. 5b, lanes 1-6). In the presence of higher Triton X-100 concentrations (1mg/mg protein) the phosphorylated band was still present with EGTA (Fig. 6, lanes and 2). The use of sodium deoxycholate to solubilise the membrane proteins results in the phosphorylation of the enzyme being abolished, whether in the presence of calcium or EGTA (Fig. 6, lanes 3 and 4).

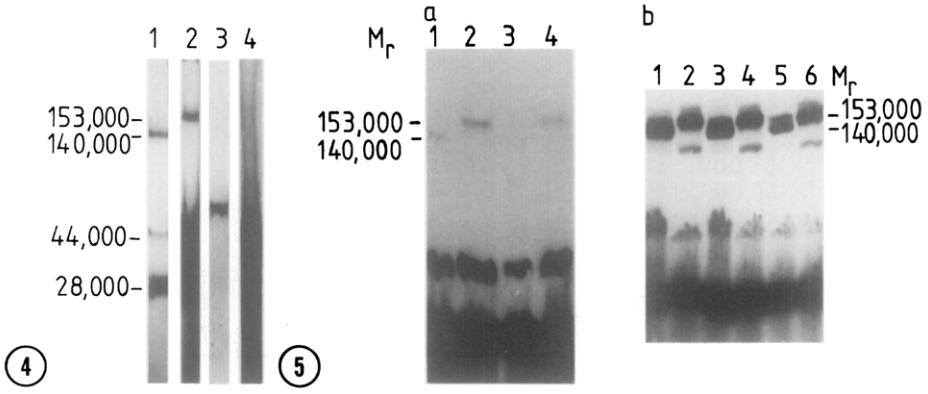

Figure 4. Effect of EGTA on microsome preparations. Autoradiograph of 5% pH 6.0 SDS polyacrylamide gel. Lane 1: microsomes phosphorylated in the presence of 50μM $LaCl_3$ for 15 s on ice; lane 2: as lane 1 but with 500μM EGTA in place of $LaCl_3$; lane 3: as lane 2 but 5mM unlabelled ATP added for 1 min after phosphorylation; lane 4: as lane 2, but pelleted membrane preparation washed with 50mM hydroxylamine in 100mM MES/KOH pH 6.0 prior to electrophoresis.

Figure 5. Formation of phosphorylated intermediates in microsomes, autoradiographs of 5% pH 6.0 SDS polyacrylamide gels. (a) After permeabilising treatments. Lane 1: microsomes phosphorylated after sonication with 0.1mg Triton X-100/mg protein in the presence of calcium; lane 2: as lane 1, but with 500μM EGTA in place of calcium; lane 3: as lane 1, but microsomes vortexed for 10 min; lane 4: as lane 3, but with 500μM EGTA. (b) After freeze/thaw treatments. Lane 1: microsomes phosphorylated after two freeze/thaw cycles in the presence of calcium; lane 2: as lane 1, but with 500μM added EGTA in place of calcium; lanes 3 and 4, as lanes 1 and 2 respectively, but microsomes phosphorylated after three freeze/thaw cycles; lanes 5 and 6: as lanes 1 and 2 respectively, but microsomes phosphorylated after four freeze/thaw cycles.

Phosphorylation of the purified enzyme preparation differed from that of the microsome preparation in that it showed calcium dependence. The band of phosphorylation at 140,000 M_r in the presence of calcium was not apparent in the presence of EGTA (fig. 2, lane 5). No band of phosphorylation at 153,000 M_r appeared and when polyacrylamide gels prepared in parallel were silver stained for protein (Fig. 7), no differences in M_r between Ca^{2+} treated and EGTA treated enzyme was apparent. (It should be noted that the presence of the lower M_r polypeptides visible in the purified enzyme preparation on pH 6.0 gels are likely to be the result of proteolysis of the purified enzyme; comparable gels under neutral conditions have been presented previously (4) and evidence for partial proteolysis is discussed therein). Phosphorylation of the erythrocyte calcium pumping ATPase is calcium sensitive (8), both in membrane preparations and in the purified enzyme and it thus appears that there may be differences between it and the plant enzyme in this respect. While it is possible that the 153,000 M_r phosphorylation is due to the presence of another ATPase in the membrane preparation which is inhibited in the presence of calcium, it is feasible that it is the calmodulin-stimulated

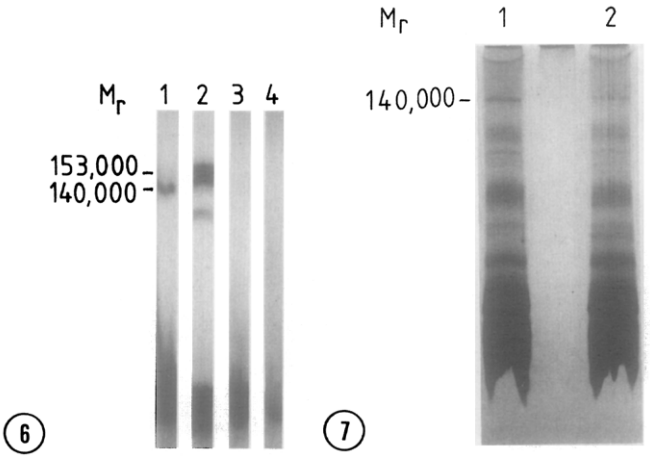

Figure 6. Phosphorylated intermediate formation by microsome preparations after solubilisation with increased detergent concentrations; autoradiograph of 5% pH 6.0 SDS polyacrylamide gel. Lane 1: membrane phosphorylated on ice for 15 s after solubilisation with 1mg Triton X-100/mg protein in the presence of 50μM added calcium, lane 2: as lane 1 but in the presence of 500μM EGTA in place of calcium; lanes 3 and 4: as lanes 1 and 2 respectively, but microsome proteins solubilised with 1mg sodium deoxycholate/mg protein.

Figure 7. Effect of EGTA on purified enzyme. Purified enzyme preparation was incubated with 500μM EGTA for 15 s on ice and precipitated with TCA. The pellet was then prepared for electrophoresis as described in 'Methods' and separated on 5% SDS polyacrylamide gels. The resulting gels were silver stained by the method of (10).

enzyme running at an anomalous M_r in the presence of EGTA. If this is the case, not only is a marked conformational change indicated (not noted in the purified erythrocyte enzyme or in the purified plant enzyme) but the membrane-associated enzyme also appears to phosphorylate in the presence of EGTA, even after extensive microsome-permeabilising treatments. As yet, an antibody which recognises the calmodulin-stimulated enzyme in microsome preparations is not available; therefore it has not been possible to locate the calmodulin-stimulated enzyme in microsomes in the presence of calcium or EGTA by any method other than by the formation of phosphorylated intermediates, and thereby to unequivocally identify the 153,000 M_r polypeptide as the calcium pump. As the purified enzyme behaves similarly to the erythrocyte enzyme in being calcium-sensitive, it appears that any differences lie in the inter-relationship between the enzyme and the membrane. Possibly the plant membrane retains sufficient strongly bound calcium to locally overcome the effect of the EGTA. Clearly, no entirely satisfactory explanation for the effect of EGTA on phosphorylation in membrane preparations will be possible until considerably more information on the membrane environment and its effects on the enzyme is obtained. It is interesting to note that Knauf et al. (12) detected a calcium-stimulated phosphoprotein in red blood cell ghosts that consisted of two components - a faint band of

approximately 142,000 M_r and a denser band of 150,000 M_r. In addition, Szasz et al. (13), studying the phosphorylation of the calcium pump in isolated erythrocyte membranes, did not show a decrease in phosphorylation in the presence of EGTA; there also appears to be a shift in the molecular mass of the enzyme in the presence of EGTA. However, in neither of these papers are the membrane preparations solubilised with detergents, as is the case in the experiments reported here.

The data presented thus further corroborates previous work (1,4) indicating that higher plant plasma membranes contain a calmodulin-dependent ATPase which strongly resembles the erythrocyte calcium pumping ATPase (4). The intact enzyme forms a phosphorylated intermediate of M_r 140,000 both in microsome preparations and when purified, in a manner anticipated for an E_1-E_2 ion translocating ATPase. The enzyme clearly differs from the higher plant endoplasmic reticulum calcium-pumping ATPase, which forms a phosphorylated intermediate of M_r 96,000 (14). Higher plant cells therefore appear to resemble mammalian cells in having two E_1-E_2 calcium pumping ATPases located in the plasma membrane and endoplasmic reticulum.

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