Calcium- and phospholipid-dependent phosphorylation of ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit by a chloroplast envelope-bound protein kinase in situ

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Phosphorylation of the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit and other polypeptides by a protein kinase bound to the chloroplast envelope in situ was inhibited by EGTA, but not by calmodulin antagonists. When the envelope membrane was extracted with 90% (v/v) cold acetone, the protein kinase activity was completely lost. The activity was restored by adding a lipid fraction extracted from the chloroplast envelope, or phospholipids such as phosphatidylserine and phosphatidylcholine. Treatment of the envelope with phospholipases decreased the protein kinase activity. This was restored by the addition of phospholipids. These results strongly suggest that the envelope-bound protein kinase is a Ca^{2+}- and phospholipid-dependent enzyme.

Protein kinase *Calcium* *Phospholipid* *Protein phosphorylation* *Chloroplast envelope* *Ribulose-1,5-bisphosphate carboxylase/oxygenase*

1. INTRODUCTION

Two Ca^{2+}- and calmodulin-dependent enzymes have been reported in the chloroplast envelope, i.e. NAD kinase [1,2] and Mg^{2+}-, Ca^{2+}- and calmodulin-stimulated ATPase [3]. The chloroplast envelope contains at least 21 proteins as separated by isoelectric focusing [4]. It is noteworthy that among the 21 envelope proteins 2 proteins are Ca^{2+}- and calmodulin-dependent enzymes. Soll and Buchanan [5] reported a cAMP-independent protein kinase in the outer envelope membrane of spinach chloroplasts. This protein kinase phosphorylated the mature form of SS and, to a lesser extent, an unidentified 24 kDa polypeptide, both of which were bound to the outer envelope membrane. Ca^{2+}- and calmodulin-dependent membrane-bound protein kinases have been separated in several plants [6–9]. Recently, a Ca^{2+}- and phospholipid-dependent protein kinase was reported in zucchini [10]. It is of interest therefore to investigate whether the protein kinase bound to the chloroplast envelope is either Ca^{2+}- and calmodulin-dependent or Ca^{2+}- and phospholipid-dependent.

Abbreviations: LS and SS, large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase

2. MATERIALS AND METHODS

2.1. *Plant and preparation of chloroplast envelope*

Spinach (*Spinacia oleracea* L.) was purchased from a local market. Intact chloroplasts were isolated and purified as in [2]. Envelope membranes were prepared as in [11].

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2.2. *Phosphorylation of polypeptides bound to the envelope*

The reaction mixture for the phosphorylation of polypeptides bound to the chloroplast envelope membrane was essentially the same as in [5]. The reaction was started by adding [γ-^{32}P]ATP (NEN) and stopped by adding trichloroacetic acid (final 12.5%, w/v). After centrifugation at 10000 × *g* for 5 min, the precipitated protein was washed twice with ethanol and then dried under vacuum. For phosphorylation of the envelope polypeptides in intact chloroplasts, a reaction mixture containing 0.33 M sorbitol was used. The reaction was stopped by adding EDTA (final 10 mM) containing 0.33 M sorbitol. The chloroplasts were osmotically ruptured and centrifuged at 10000 × *g* for 1 min to sediment the thylakoids. The supernatant was recentrifuged at 150000 × *g* for 1 h to obtain the envelopes. The pellet was washed and dried as above.

The dried envelope membranes were dissolved in the sample buffer of SDS-polyacrylamide gel electrophoresis, and the phosphorylated polypeptides were analyzed in 12.5 or 15% (w/v) acrylamide gel as in [12]. The gels were stained with Coomassie blue, destained and autoradiographed.

2.3. *Extraction of lipids from the envelope membrane*

The envelope membranes were treated with 90% (v/v) acetone at 0°C for 10 min. The mixture was centrifuged at 10000 × *g* for 1 min and the yellow supernatant concentrated in a rotary evaporator to remove the acetone and used as the lipid fraction. The precipitate was resuspended in 10 mM Hepes-KOH buffer (pH 7) and recentrifuged at 150000 × *g* for 30 min. The supernatant was discarded and the precipitate resuspended in the above buffer was used as the lipid-depleted envelope fraction. The volume of both the lipid fraction and the lipid-depleted envelope was adjusted to the volume of the original envelope suspension.

2.4. *Phospholipase treatment of the envelope*

The envelope suspension was incubated in 10 mM Hepes-KOH buffer (pH 7) with either *Bacillus cereus* phospholipase C (200 units/ml), hog pancreas phospholipase A_2 (200 units/ml) or cabbage phospholipase D (1.2 units/ml). After treatment, phosphorylation was started by adding the reaction mixture for phosphorylation. Phospholipases were from Boehringer.

3. RESULTS

3.1. *Calcium-dependent phosphorylation of the chloroplast envelope polypeptides*

When the envelope membranes were incubated with [γ-^{32}P]ATP and Mg^{2+}, the SS and a 25 kDa polypeptide which may correspond to the 24 kDa polypeptide in [5] were strongly phosphorylated as reported in [5] (fig.1). Six polypeptides of >30–70 kDa and several polypeptides of 70 kDa were also phosphorylated. Phosphorylation was slightly stimulated by $CaCl_2$ (fig.1, lane 2). Addition of calmodulin alone (lane 3) or with $CaCl_2$ (lane 4) had essentially no effect, but 1 mM EDTA completely inhibited phosphorylation (lane 5). The calmodulin antagonists compound 48/80 (Sigma) (lane 6) and calmidazolium (Boehringer) (lane 7) did not inhibit phosphorylation in the presence of $CaCl_2$ and calmodulin, suggesting that the chloroplast envelope-bound protein kinase is Ca^{2+}- but not calmodulin-dependent.

Fig.2 shows the effect of Ca^{2+} concentration on the phosphorylation of the SS [5] by the envelope-bound protein kinase. Maximum activation was achieved at 20 μM free Ca^{2+}, while higher Ca^{2+} concentrations were inhibitory.

3.2. *Phospholipid-dependent phosphorylation of the envelope polypeptides*

When envelope membranes were extracted with 90% (v/v) acetone, the protein kinase activity was completely lost (fig.3, lane 1). The enzyme activity of the lipid-depleted membrane was restored by adding back the lipid fraction extracted from the envelope membrane (lanes 2 and 3). The lipid fraction itself had no protein kinase activity (lane 6). Phosphatidylcholine and phosphatidylserine were also effective in restoring enzyme activity but to a lesser extent than the lipid fraction (lanes 4 and 5). The recovered activities were Ca^{2+}-dependent (not shown). These results suggest that the envelope-bound protein kinase is Ca^{2+}- and phospholipid-dependent.

The phospholipid dependency of protein kinase was confirmed by treatment with phospholipases. Protein kinase activity was decreased in a time-dependent manner when envelopes were incubated

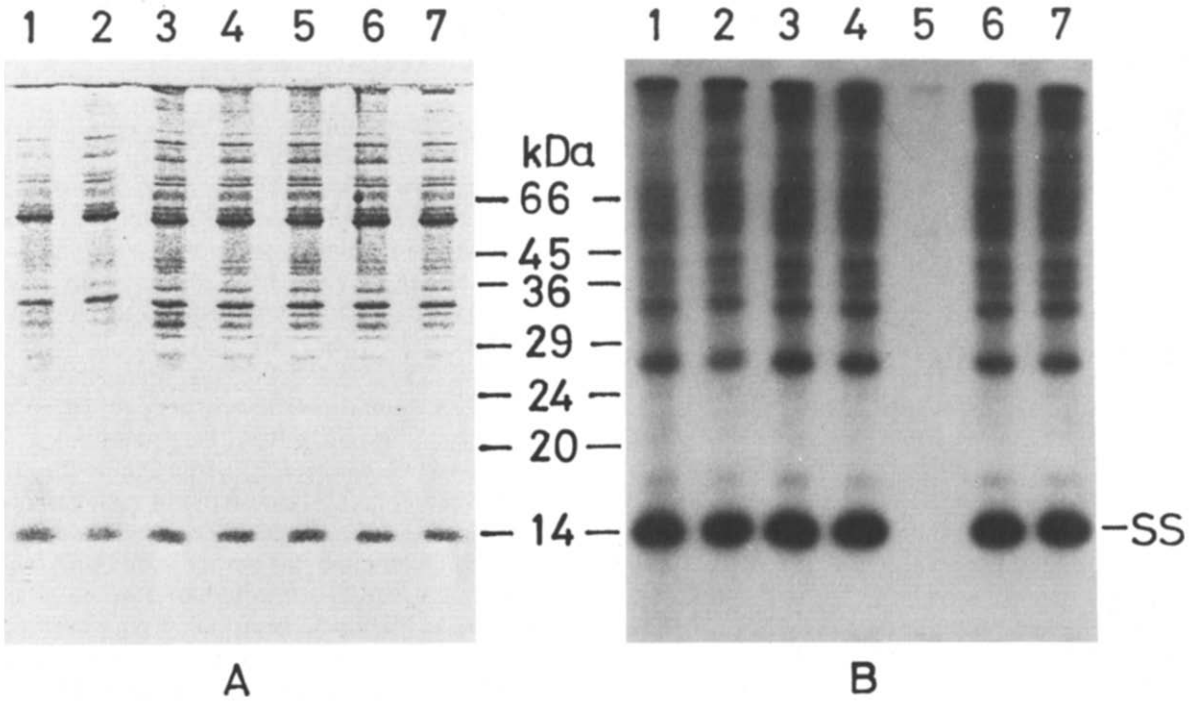

Fig.1. Phosphorylation of chloroplast envelope polypeptides by envelope-bound protein kinase. The reaction mixture contained 50 mM Hepes-KOH (pH 7), 4 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP, 100 μg/ml envelope protein (lane 1), and 20 μM $CaCl_2$ (lane 2), 0.1 μM spinach calmodulin (lane 3), 20 μM $CaCl_2$ and 0.1 μM calmodulin (lane 4), 20 μM $CaCl_2$, 0.1 μM calmodulin and 1 mM EGTA (lane 5), 20 μM $CaCl_2$, 0.1 μM calmodulin and 5 μg/ml compound 48/80 (lane 6), 20 μM $CaCl_2$, 0.1 μM calmodulin and 5 μM calmidazolium (lane 7). A, protein staining; B, autoradiogram. Numbers between A and B denote molecular mass markers.

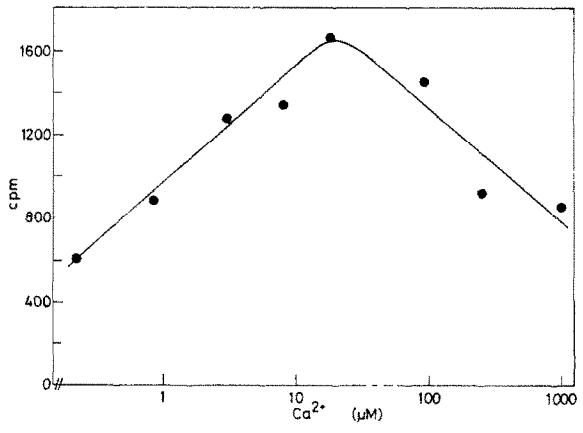

Fig.2. Effect of Ca^{2+} concentration on phosphorylation of SS by envelope-bound protein kinase. Phosphorylation was carried out at the indicated concentration of free Ca^{2+}. The radioactivities in the SS were determined by liquid scintillation counting after SDS gel electrophoresis.

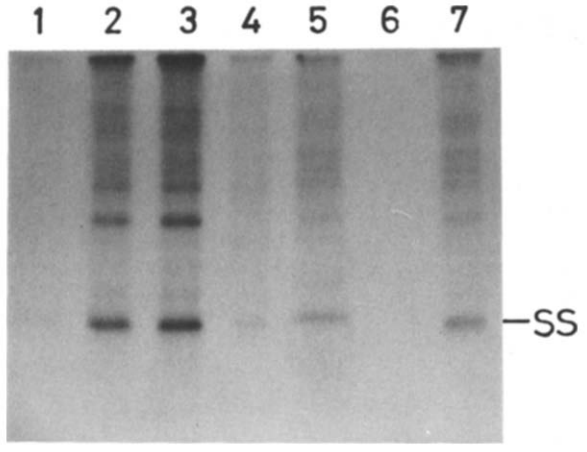

Fig.3. Effect of acetone treatment on the activity of envelope-bound protein kinase. Lanes 1–5, acetone-treated envelopes. Additions: 20 μl lipid fraction (lane 2), 40 μl lipid fraction (lane 3), 4 mg/ml phosphatidylcholine (lane 4), 4 mg/ml phosphatidylserine (lane 5). Lane 6, 40 μl lipid fraction minus envelopes. Lane 7, envelopes not treated with acetone.

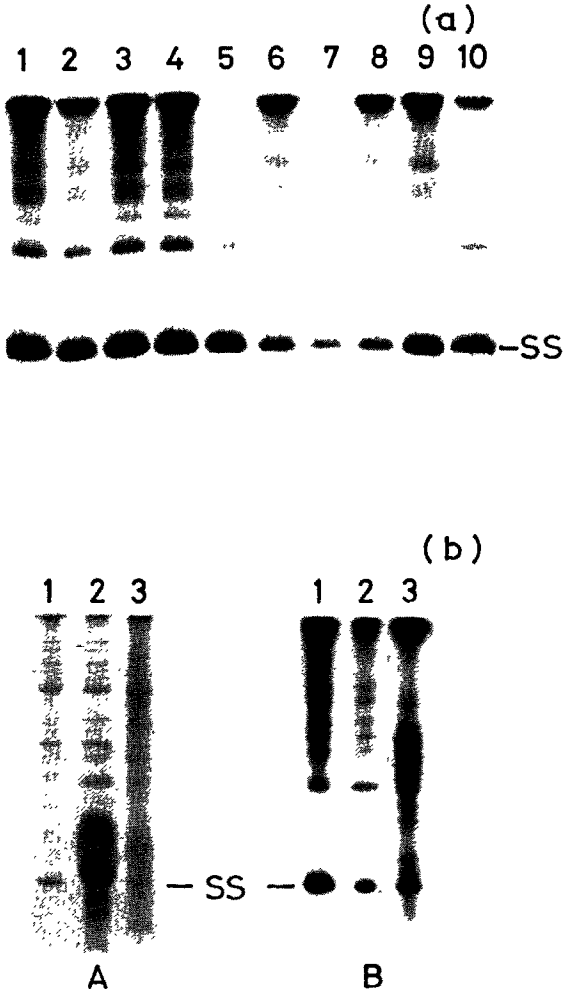

Fig.4. Effect of phospholipase treatment on the envelope-bound protein kinase activity. (a) Envelopes were treated at 25°C for 0, 5, 10 and 20 min in the absence (lanes 1–4) and presence (lanes 5–8) of phospholipase C. Lane 9, 4 mg/ml phosphatidylserine was included during 20 min phospholipase treatment. Lane 10, 4 mg/ml phosphatidylserine was added after 20 min treatment. (b) Lane 1, control; lane 2, phospholipase A_2-treated; lane 3, phospholipase D-treated. A, protein staining; B, autoradiogram.

with phospholipase C (fig.4a, lanes 5–8). The decreased protein kinase activity was restored by phosphatidylserine addition (lane 9). Protein kinase inactivation by phospholipase C was prevented by including phosphatidylserine during enzyme treatment (lane 10). Similar results were obtained with phospholipase A_2 and phospholipase D (fig.4b, lanes 2 and 3).

3.3. *Phosphorylation of the envelope polypeptides in intact chloroplasts*

Since phosphorylation of the envelope polypeptides was studied with isolated membranes, the possibility that phosphorylation was an artifact caused by breaking the chloroplasts could not be excluded. Phosphorylation was thus examined using intact chloroplasts. As shown in fig.5, the SS and 25 kDa polypeptide were also phosphorylated in whole chloroplasts. As with isolated envelopes, phosphorylation in the intact chloroplasts was inhibited by EGTA (lane 5), while the addition of $CaCl_2$ (lane 2), $CaCl_2$ and calmodulin (lane 3) and calmidazolium (lane 4) had no effect. The number of polypeptides phosphorylated in the intact chloroplasts was less than that of the isolated envelope, while a 35 kDa polypeptide was observed only in the intact chloroplasts. Polypeptides phosphorylation in the intact chloroplasts may reflect in vivo events. Phosphorylation of the 25 kDa polypeptide in the intact chloroplasts was

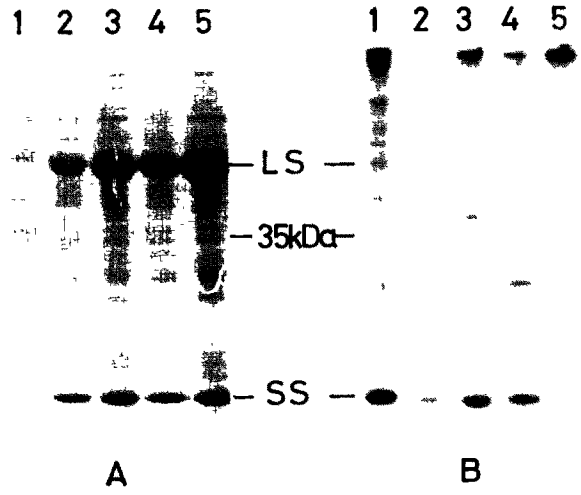

Fig.5. Phosphorylation of envelope-bound polypeptides in intact chloroplasts. Lane 1, isolated envelopes; lanes 2–5, intact chloroplasts. Protein kinase was assayed in the presence of 20 μM $CaCl_2$ (lane 1 and 2), 20 μM $CaCl_2$ plus 0.1 μM calmodulin (lane 3), 5 μM calmidazolium (lane 4) and 1 mM EGTA (lane 5). Amounts of protein applied to lanes 2 and 4 were about half of those in lanes 3 and 5. A, protein staining; B, autoradiogram.

not inhibited by EGTA, but the reason for this is obscure.

4. DISCUSSION

This paper provides clear evidence that the protein kinase bound to the chloroplast envelope is a Ca^{2+}- and phospholipid-dependent enzyme. The main protein substrate for this enzyme is the mature form of SS which was reported to be tightly bound to the envelope membrane [13]. An ATP requirement for the transport of the cytoplasmically synthesized precursor of SS as well as other polypeptides across the envelope membrane has been established [14,15]. The possibility that phosphorylation occurs in the transport process of the precursor of SS has been suggested [5], however, there is no evidence to support this. The involvement of another Ca^{2+}-dependent enzyme in the envelope, Mg^{2+}-, Ca^{2+}- and calmodulin-stimulated ATPase, in the ATP-requiring transport of cytoplasmically synthesized proteins was also postulated [3]. It is tempting to associate these 2 Ca^{2+}-dependent enzymes with the transport of cytoplasmically synthesized polypeptides into chloroplasts. However, further studies are required to evaluate this hypothesis.

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