The phosphatidic acid phosphatase of the chloroplast envelope is located on the inner envelope membrane

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The envelope from spinach chloroplasts contains an alkaline phosphatidic acid phosphatase which was found to be located on the inner envelope membrane. The diacylglycerol formed by this enzyme from endogenous phosphatidic acid is then used as a substrate for galactolipid synthesis on the inner envelope membrane.

Spinach *Chloroplast envelope* *Phosphatidic acid phosphatase* *Galactosyltransferase*

1. INTRODUCTION

The chloroplast envelope membranes contain all the enzymes involved in phosphatidic acid and diacylglycerol synthesis. For instance, the envelope membranes from spinach [1–3] or pea [3] chloroplasts contain a specific alkaline, membrane-bound phosphatidic acid phosphatase (or phosphatidate phosphohydrolase, EC 3.1.3.4) which dephosphorylates phosphatidic acid to diacylglycerol. Both phosphatidic acid and diacylglycerol are key metabolites for glycerolipid biosynthesis in chloroplasts. Diacylglycerol is the substrate of a UDP-galactose:diacylglycerol galactosyltransferase, specifically associated with the envelope membranes [4], which synthesize monogalactosyldiacylglycerol (MGDG), the major chloroplast polar lipid. Diacylglycerol may also serve as a substrate for sulfolipid synthesis, which probably takes place in isolated intact chloroplasts [5,6]. In addition to its importance in diacylglycerol synthesis, phosphatidic acid can be used for phosphatidylglycerol synthesis: indeed, the synthesis of the major chloroplast phospholipid has been clearly demonstrated to occur in isolated intact chloroplasts [6,7]. These observations are strengthened by the fact that phosphatidic acid and diacylglycerol synthesized within isolated envelope membranes from either [^{14}C]acetate [8] or a mixture of different acyl-CoA and/or acyl-ACP species [3] have a specific fatty acid pattern (i.e., with C18 fatty acids and C16 fatty acids respectively, on the *sn*-1 and *sn*-2 position of the glycerol backbone). This pattern is identical to that found in MGDG from 16:3-containing plants and in sulfolipids and phosphatidylglycerol synthesized either in vivo [9–12] or in vitro [6,7,13,14]. Therefore, it is important to localize precisely the enzymes involved in diacylglycerol biosynthesis in order to understand their role in plastid glycerolipid metabolism.

We report here that the envelope phosphatidic acid phosphatase is actually localized on the inner membrane of the chloroplast envelope. Preliminary accounts of these findings have been presented previously [15].

2. MATERIALS AND METHODS

2.1. *Isolation of spinach chloroplasts and envelope membranes*

Intact chloroplasts were isolated from 3–4 kg spinach leaves by differential centrifugation followed by Percoll density gradient centrifugation [16]. Envelope, stroma and thylakoids were prepared from intact, purified chloroplasts as

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described by Douce et al. [17]. Membrane fractions enriched in outer and inner envelope membranes, respectively, were prepared as follows [18]: intact, purified spinach chloroplasts (150–200 mg chl) were kept on ice for 10 min in 60 ml (2 × 30 ml, final volume) of a hypertonic medium containing 0.6 M mannitol, 4 mM $MgCl_2$ and 10 mM Tricine–NaOH (pH 7.9). Under these conditions, the outer envelope membrane of the chloroplasts appears to be loosely attached to the inner envelope membrane with large empty spaces in between [19]. Chloroplast suspension (30 ml) was then placed in a cold (0–4°C) Yeda press (Linca, Tel Aviv). The cell's pressure was then raised to 5 bar, using nitrogen. The shrunken chloroplasts were then extruded through the aperture of the Yeda press at a speed of 10 ml/min. The suspension thus obtained was then centrifuged for 10 min at 10000 rev/min (Sorvall RC5, SS 34 rotor). The pellet, containing intact and broken chloroplasts, was discarded and the supernatant diluted twice with 10 mM Tricine–NaOH (pH 7.9) and 4 mM $MgCl_2$ to give a suspension containing 0.3 M mannitol (final concentration). 13 ml aliquots of the suspension (2–5 μg chl/ml; 1–2 mg protein/ml) were layered on top of discontinuous sucrose gradients (1 M, 0.65 M and 0.4 M) and centrifuged for 90 min at 23000 rev/min (Beckman L2 65 B, SW 27 rotor). The two yellowish bands recovered at the 1 M/0.65 M interface (heavy fraction, d = 1.13 g/cm^3, or fraction 3, see [18]) and at the 0.65 M/0.4 M interface (light fraction, d = 1.08 g/cm^3, or fraction 2, see [18]), respectively, were removed from the tubes with a Pasteur pipette, diluted 4 times with 10 mM Tricine–NaOH (pH 7.9), 4 mM $MgCl_2$ and spun for 1 h at 26000 rev/min (Beckman L2 65B, SW 27 rotor). The pellets were suspended in a medium containing 0.3 M sucrose and 10 mM Tricine–NaOH (pH 7.9). Analyses of the chemical components of these fractions (proteins, lipids, pigments) and of their enzymatic activities led us to conclude that the light and the heavy fractions were enriched in outer and inner envelope membranes, respectively [18,20].

2.2. *Assay of phosphatidic acid phosphatase*

Phosphatidic acid phosphatase was measured as follows. First, [^{14}C]phosphatidic acid was synthesized in the membrane fractions in a 600 μl reaction mixture containing 10 mM Mops–NaOH (pH 7.0), 8 mM ATP, 0.7 mM coenzyme A, 8 mM $MgCl_2$, 1 mM oleoyl-CoA, 1 mM palmitoyl-CoA, 1 mM *sn*-[^{14}C]glycerol 3-phosphate (5 μCi/μmol) and enzyme: 0.3–0.5 mg membrane protein from the light or heavy fraction and 5 mg protein from a chloroplast extract prepared as in [17]. At pH 7.0, the activity of the envelope phosphatidic acid phosphatase is low, therefore, mostly phosphatidic acid accumulates in the membrane [1,2]. After 1 h incubation, the membrane vesicles loaded with [^{14}C]phosphatidic acid were separated from the soluble components by centrifugation at 3°C through 0.3 M sucrose containing 10 mM Tricine–NaOH (pH 7.0), 10 mM EDTA. After centrifugation for 1 h at 45000 rev/min (Beckman L2 65B, SW 50 rotor), the membrane pellets were suspended in the following medium: 0.3 M sucrose, 20 mM Tricine–NaOH (pH as indicated, usually 9.0), final protein concentration 0.5–0.9 mg/ml. Under these conditions, an increase in the pH triggers phosphatidic acid hydrolysis [1]. Phosphatidic acid phosphatase activity was measured by following the appearance of [^{14}C]diacylglycerol and/or disappearance of [^{14}C]phosphatidic acid from the membrane fractions at room temperature. At various times, 80 μl aliquots containing 40–70 μg membrane protein were withdrawn for lipid analyses. The reaction was then terminated, the lipids extracted and analyzed as described in [1].

3. RESULTS AND DISCUSSION

When phosphatidic acid phosphatase activity in the light and heavy fractions, respectively, was assayed under the conditions previously described for the envelope phosphatidic acid phosphatase (i.e., at alkaline pH), we observed that practically no hydrolysis of phosphatidic acid occurred with the light fraction (fig.1A). In marked contrast, with the heavy fraction, phosphatidic acid hydrolysis was very active (fig.1B). Under these conditions, the loss of [^{14}C]phosphatidic acid was associated with the stoichiometric appearance of [^{14}C]diacylglycerol (fig.1B). Since the light and the heavy fractions analysed are, respectively, enriched in outer and inner envelope membranes from chloroplasts [18,20], these results demonstrate that the alkaline phosphatidic acid phosphatase is

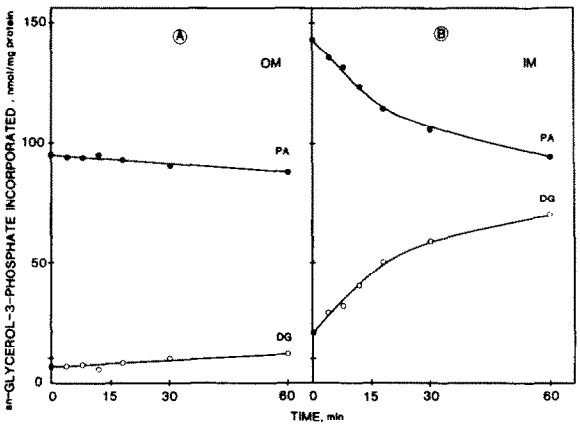

Fig.1. The appearance of diacylglycerol during endogenous phosphatidic acid (PA) hydrolysis by isolated membrane fractions enriched (A) in outer (OM) and (B) in inner (IM) envelope membranes from spinach chloroplasts. Assay conditions are described in section 2.

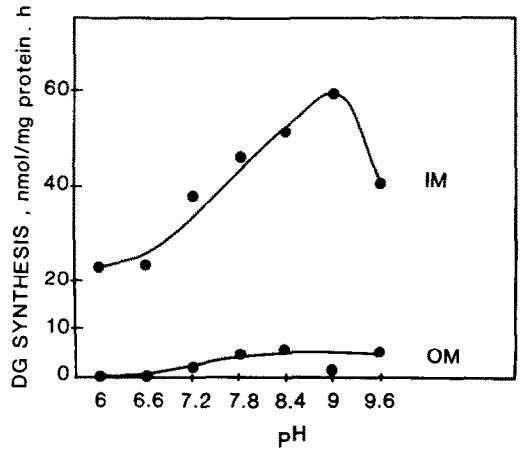

Fig.2. Effect of pH on the rate of endogenous phosphatidic acid hydrolysis by isolated membrane fractions enriched in outer or inner envelope membranes, respectively, from spinach chloroplasts. Assay conditions are described in section 2. The amount of [^{14}C]diacylglycerol at zero time of the incubation was subtracted from all other values. For abbreviations, see fig.1.

located on the inner membrane of spinach chloroplasts.

However, the lack of activity of phosphatidic acid phosphatase in the membrane fraction enriched in outer envelope membrane could be due to the experimental conditions used. As demonstrated in [1], the envelope phosphatidic acid phosphatase has an alkaline pH optimum (9.0) and is inhibited by divalent cations (such as $MgCl_2$). Therefore, the enzyme, if present in the outer envelope membrane, could have a different pH optimum and a different cation requirement. Fig.2 demonstrates that there was no stimulation of the rate of phosphatidic acid hydrolysis in the light fraction at neutral or acidic pH. Fig.2 also shows that the optimum pH value was identical in the heavy fraction to the value previously found in the whole envelope membranes [1]. In addition, we demonstrated that $MgCl_2$ (10 mM) had an inhibitory effect (70–80% inhibition) on phosphatidic acid hydrolysis in both fractions analyzed.

These results demonstrate that the outer envelope membrane probably does not contain a phosphatidic acid phosphatase activity. The small activity found in the light fraction was entirely attributable to inner membrane contamination. In fact, using rocket immunoelectrophoresis of the light and heavy fractions in agarose gels containing antibodies specific for the outer or inner envelope membrane polypeptides [18], we demonstrated that the light fraction contains about 90% outer membrane protein whereas the heavy fraction contains about 80% inner membrane protein [18,20]. As shown previously [20], the ratio of activities found in the light fraction (7 ± 1 nmol diacylglycerol formed/h per mg protein) to those found in the heavy fraction (75 ± 20 nmol diacylglycerol formed/h per mg protein) is very close to those obtained with other enzymatic activities associated with the inner envelope membrane such as acyl-CoA thioesterase or UDP-galactose:diacylglycerol galactosyltransferase [20,21]. Thus, we can conclude that the inner envelope membrane from spinach chloroplasts contains an active alkaline phosphatidic acid phosphatase, which is inhibited by $MgCl_2$, and is, therefore, identical with the enzyme previously characterized in whole envelope membranes [1].

As mentioned earlier, in intact chloroplasts, as well as in isolated envelope membranes, diacylglycerol is used as a substrate in the production of MGDG. The UDP-galactose:diacylglycerol galactosyltransferase which catalyses the transfer of the galactose moiety of UDP-galactose to endogenous diacylglycerol is a characteristic

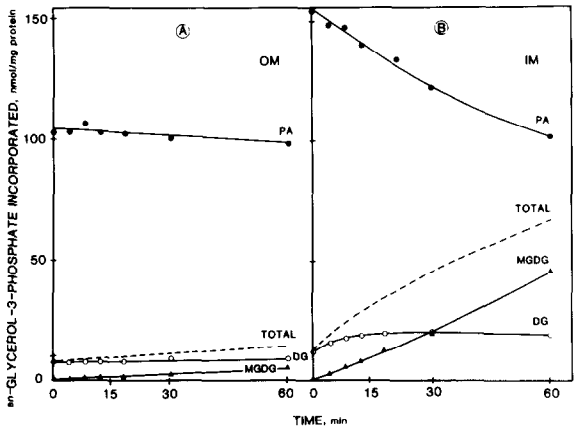

Fig.3. The appearance of diacylglycerol during endogenous phosphatidic acid hydrolysis by isolated membrane fractions enriched (A) in outer envelope membrane, and (B) in inner envelope membrane from spinach chloroplasts. Assay conditions are described in section 2. For abbreviations, see fig.1.

marker for envelope membranes [4,8]. In addition, we have previously demonstrated that this enzyme is associated with the inner envelope membrane from spinach chloroplasts [20,21]. In contrast, the authors in [22] have shown that in pea chloroplasts, this activity is located on the outer membrane. However, the galactosyltransferase activity, which does require divalent cations and has a pH optimum above 7.5 [1], and the phosphatidic acid phosphatase have almost identical optimum conditions. Therefore, experiments were undertaken to show that endogenously labeled phosphatidic acid could be converted to MGDG by the sequential action of the envelope phosphatidic acid phosphatase and UDP-galactose:diacylglycerol galactosyltransferase. Fig.3 demonstrates that endogenously labeled phosphatidic acid was rapidly incorporated into MGDG after addition of UDP-galactose to the incubation mixture only with inner membrane-rich fraction. When thermolysin-treated chloroplasts were used to prepare membrane fractions enriched in outer and inner envelope membranes, respectively, the rate of disappearance of labeled diacylglycerol molecules from the membrane fractions was much higher (not shown). It has been shown that thermolysin treatment of intact chloroplasts destroys the galactolipid:galactolipid galactosyltransferase [15,23] which is responsible for the accumulation of diacylglycerol in isolated envelope membranes. Thus, in the case where the two envelope membranes were prepared from thermolysin-treated chloroplasts, the newly synthesized, labeled diacylglycerol molecules were not diluted by the large preexisting pool of diacylglycerol, as shown in [15,23].

The results here give further support for the localisation on the inner envelope membrane of the UDP-galactose:diacylglycerol galactosyltransferase. Indeed, such a localisation was expected since:

(i) fatty acids used for galactolipid synthesis are formed in the stroma of chloroplasts [24];
(ii) acyl-ACP are probably the in vivo acyl donors for galactolipids [3].

Thus, the differences between our data and those in [22] are unexpected but could be due to species specificities, as discussed in [20].

Finally, one of the most interesting questions which remains to be elucidated is how phosphatidic acid can be synthesized in the outer and in the inner envelope membrane. The fact that a soluble fraction is necessary for both membranes to form phosphatidic acid demonstrates that both membranes are unable to form lysophosphatidic acid. Therefore, the enzyme responsible for the synthesis of lysophosphatidic acid could be localized in the intermembrane space of the chloroplast envelope (suggested in [2]). Another possibility is that the inner membrane can use lysophosphatidic acid formed on the stroma side of the inner envelope membrane whereas the outer membrane uses lysophosphatidic acid formed on the cytoplasmic side of the outer envelope membrane. In both cases, the origin and the nature of fatty acid thioester derivatives used is not clear. Further work has to be done in order to elucidate this problem.

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