CHARACTERIZATION OF PHOSPHATIDATE PHOSPHOHYDROLASE ACTIVITY ASSOCIATED WITH CHLOROPLAST ENVELOPE MEMBRANES

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1. Introduction

Intact purified chloroplasts from spinach contain two acyltransferases [1]. One is a soluble enzyme [2,3] and catalyses the acylation of *sn*-glycerol 3-phosphate in the C-1 position [4], the final product is lysophosphatidic acid. This enzyme is probably loosely bound to the envelope membranes and is released in the medium during envelope isolation [3]. The second acylase is an acyl-CoA:acyl *sn*-glycerol 3-phosphate acyltranferase which is firmly bound to the envelope membranes and forms phosphatidic acid [3]. Consequently, purified intact spinach chloroplasts synthesize phosphatidic acid and this synthesis occurs almost exclusively in the envelope membranes [3].

The purpose of this paper is to report the presence of a specific alkaline phosphatidate phosphohydrolase (EC 3.1.3.4.) in spinach chloroplast envelope membranes. The activity of this enzyme occupies a central role in the biosynthesis of glycerophospholipids. Indeed the hydrolysis of phosphatidic acid, giving rise to *sn*-1,2-diacylglycerols, constitutes the first branch point in the biosynthesis of galactolipids and phospholipids.

2. Materials and methods

Chloroplasts were isolated from month 2 spinach leaves using the classical method [5]. In order to avoid 'microsomes', swollen grana lamellae and plastoglobuli clusters in the chloroplast fraction, the intact chloroplasts were purified by density gradient centrifugation either in Percol™ [6] or sucrose [7]. From 2 kg leaves the yield of intact and purified chloroplasts is equivalent to ~30–50 mg chlorophyll (500–900 mg chloroplastic proteins).

Envelope and thylakoid fractions were prepared from intact purified spinach chloroplasts as in [8]. We have systematically verified that NADH:cytochrome *c* oxidoreductase activity is negligible in the envelope fraction. In addition the envelope is devoid of *b*-type cytochromes. These findings preclude the possibility of significant contamination of the envelope fraction by extra-chloroplastic membranes. From 2 kg leaves the yield of envelope membranes is equivalent to ~20 mg proteins.

Assay of phosphatidate phosphohydrolase: In the first step the isolated envelope membranes were loaded with [^{14}C]phosphatidic acid. The complete reaction medium contained: 10 mM tricine–NaOH buffer (pH 7.6); 0.3 M sucrose; 4 mM ATP; 0.2 mM coenzyme A; 1 mM $MgCl_2$; 1 mM *sn*-[^{14}C]glycerol 3-phosphate (spec. act. 2.2×10^8/dpm/mmol) and known amounts of chloroplast extract [3] and envelope membranes in 3.6 ml final vol. Under these conditions, the envelope membranes which contain an acyl-coenzyme A synthetase [3,9] synthesized phosphatidic acid very actively. After 1 h incubation the reaction mixture was diluted twice with 10 mM tricine–NaOH buffer (pH 7.6) containing 5 mM EDTA. Envelope membranes loaded with [^{14}C]-phosphatidic acid were then separated from soluble components by centrifugation through 0.5 M sucrose containing 10 mM tricine–NaOH buffer (pH 7.6). After 30 min at 22 000 rev./min (Beckman SW 40) the envelope pellet was suspended in the following medium: 0.3 M sucrose; 10 mM tricine–NaOH buffer

(pH as indicated), final protein conc 10 mg/ml In the second step, phosphatidate phosphohydrolase activity was measured by following the appearance of [^{14}C]diacylglycerol and/or disappearance of [^{14}C]-phosphatidic acid in envelope lipids Incubation temperature was 25°C At various times 200 μl aliquots containing 2 mg envelope proteins (3 mg envelope lipids) were taken for lipid analysis In order to end the reaction and simultaneously to extract the lipids, 750 μl $CH_3OH/CHCl_3$ (2 1, v/v) were added to give a monophasic solution After 2 min, 250 μl $CHCl_3$ and 250 μl H_2O were added and the mixture was agitated by bubbling with N_2 and then centrifuged A suitable portion of the $CHCl_3$ layer was chromatographed on silica gel-precoated thin-layer chromatography plates (Merck) in solvent system $CHCl_3$/CH_3OH/H_2O (65 25 4, v/v/v) The radioactivity was located by autoradiography (Kodirex film, Kodak) The radioactive area ([^{14}C]phosphatidic acid, [^{14}C]-diacylglycerol and [^{14}C]monogalactosyldiacylglycerol) were scraped from the plate into counting vials and 10 ml 'Aquasol cocktail' (New England Nuclear) were added The vials were counted by liquid scintillation (Intertechnique SL 4000) In order to characterize phosphatidic acid and diacylglycerol the lipids were hydrolyzed by the mild alkaline procedure in [10] and the deacylated products (*sn*-glycerol 3-phosphate and glycerol) were chromatographed in two dimensions on paper (Whatman no 2) A solvent system of phenol/H_2O (100 38, v/v) was used in the first development and $CH_3OH/HCOOH/H_2O$ (80.13 7, v/v/v) in the second development

ATP (sodium salt), coenzyme A (trilithium salt from yeast) and UDP-galactose were purchased from Sigma *sn*-[U-^{14}C]glycerol 3-phosphate (disodium salt, spec act 220 Ci/mol) was purchased from New England Nuclear and was diluted to the required specific activity with *sn*-glycerol 3-phosphate (Sigma) Phosphatidic acid from corn seeds was a generous gift from Dr Faure Pasteur Institute, Paris)

Protein concentrations were determined by the Lowry method [11] with bovine serum albumin as standard

3. Results and discussion

When purified chloroplast envelope membranes loaded with [^{14}C]phosphatidic acid were incubated at 25°C and at pH 7 0 practically no hydrolysis of phosphatidic acid occurred In marked contrast we have observed that at higher pH, hydrolysis of phosphatidic acid started readily The maximum rate of disappearance of [^{14}C]phosphatidic acid was obtained at pH 9 0 (0 6 nmol phosphatidic acid was hydrolyzed min^{-1} mg $protein^{-1}$) (fig 1) Under these conditions, the loss of [^{14}C]phosphatidic acid was associated with a stoichiometric appearance of [^{14}C]-diacylglycerol (fig 2A)

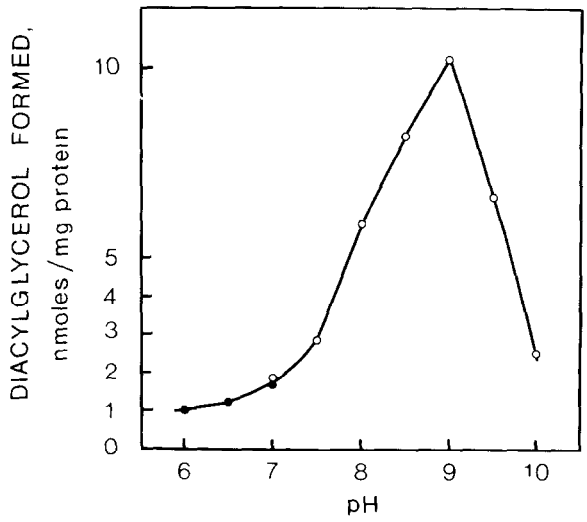

Fig 1 Effect of pH on the rate of endogenous phosphatidic acid hydrolysis by isolated spinach chloroplast envelope membranes The assay conditions were described under section 2 Assays were for 30 min The amount of [^{14}C]-diacylglycerol in zero time incubations was substracted from all assay reactions (•) Tris–maleate buffer (○) Tricine–NaOH buffer

No metal-ion requirement could be detected for the envelope phosphatidate phosphohydrolase It has to be stressed that the surface of the envelope membrane vesicles which is strongly negatively charged is able to bind large amounts of metal-ions especially Mg^{2+} [12] Consequently it is possible that this amount of bound Mg^{2+} could be sufficient to activate the envelope phosphatidate phosphohydrolase However this is unlikely for the following reasons

(i) The addition of 5 mM Mg^{2+} in the incubation medium inhibited the phosphatase by 75% (fig 3)

(ii) The addition of a cation chelating agent such as EDTA in the incubation medium increased the

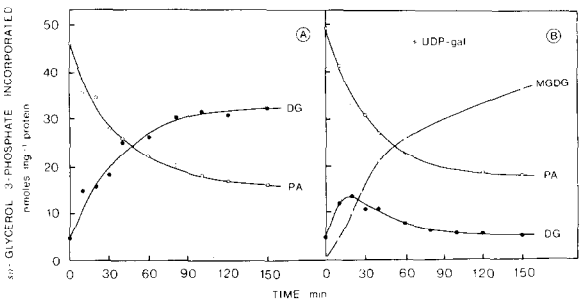

Fig.2. Diacylglycerol (DG) and monogalactosyldiacylglycerol (MGDG) appearance during the course of endogenous phosphatidic acid (PA) hydrolysis by isolated spinach chloroplast envelope membranes. (A) Without UDP-galactose; (B) with 1 mM UDP-galactose. The assay conditions (pH 8.5) were described under section 2.

rate of disappearance of [^{14}C]phosphatidic acid in envelope lipids (fig.3).

These observations rather suggest that divalent cations inhibit the envelope alkaline phosphatidate phosphohydrolase.

It is interesting to note that phosphatidic acid added exogenously to the incubation mixtures as a sonicated suspension in the reaction buffer, is hydrolyzed at an insignificant rate (result not shown). This is in contrast with endogenous phosphatidic acid which is actively synthesized by the envelope membranes. Consequently it is very likely that the exogenously added phosphatidic acid is not directly accessible to the membrane bound phosphatidate phosphohydrolase.

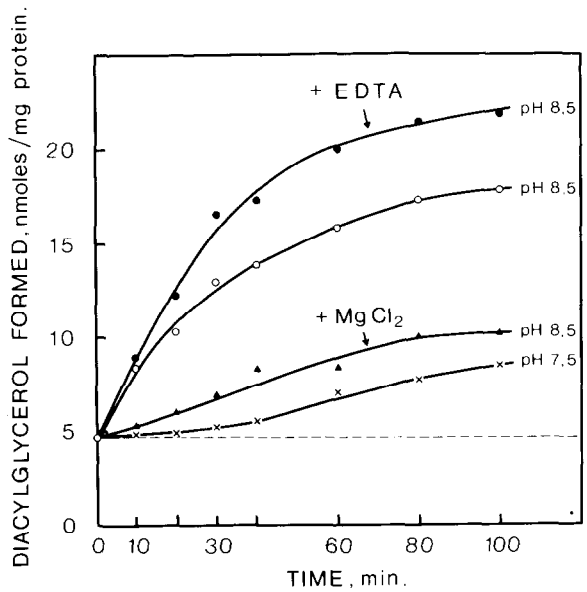

Fig.3. Effect of 5 mM EDTA and 5 mM $MgCl_2$ on the rate of endogenous phosphatidic acid hydrolysis by isolated spinach chloroplast envelope membranes. The assay conditions were described under section 2.

As mentioned earlier, in intact chloroplasts, diacylglycerol is used as a substrate in the production of monogalactosyldiacylglycerol (MGDG). Galactosyltransferase activity which catalyzed the transfer of galactose from UDP-galactose to endogenous diacylglycerol (UDP-galactose:diacylglycerol galactosyltransferase) was found in the envelope membranes [4,13]. The transferase activity which does not require divalent cation presents a pH optimum above pH 7.5 [8]. Consequently, the optimal conditions for the phosphatase and the transferase are identical. Experiments to show that endogenous-labeled phosphatidic acid could be converted to MGDG by the sequential action of the phosphatase and the galactosyltransferase were successful. As a matter of fact endogenous-labeled phosphatidic acid is converted rapidly to MGDG at pH 8.5 after addition of UDP-galactose in the incubation medium (fig.2b).

The results reported here demonstrate for the first time in plants the presence of a membrane-bound alkaline phosphatidate phosphohydrolase. It is interesting to note that this phosphatase behaves exactly like the phosphatidate phosphohydrolase found recently in the cytoplasmic membrane of *Bacillus subtilis* [14]. No metal-ion requirement could be detected for either of the 2 enzymes and both phosphatases exhibit the same pH optimum.

Finally, with the characterization of the phosphatidate phosphohydrolase in the chloroplast envelope membranes, the major reactions for biosynthesis of MGDG have now been demonstrated in envelope membrane preparations of spinach chloroplasts. One of the most interesting questions which remains to be answered is how this enzymatic pathway is regulated.

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