The galactolipid:galactolipid galactosyltransferase is located on the outer surface of the outer membrane of the chloroplast envelope

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1. INTRODUCTION

It is well known that the plastid envelope is involved in the synthesis of galactolipids (for a review see [1]). For instance it has been demonstrated that at least two distinct enzymes responsible for the synthesis of monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) are associated with chloroplast envelope membranes. The first enzyme, UDP-galactose:diacylglycerol galactosyltransferase, catalyses the incorporation of galactose from UDP-galactose into MGDG [3–5]:

Diacylglycerol + UDP-galactose → MGDG + UDP (1)

Since the isolated envelope membranes contain saturating concentrations of diacylglycerol, the addition of UDP-galactose to isolated envelope membrane vesicles triggers MGDG synthesis at the expense of the endogenous diacylglycerol pool [2]. The second enzyme synthesizes DGDG and is either a UDP-galactose:MGDG galactosyltransferase [3–5]:

MGDG + UDP-galactose → DGDG + UDP (2)

or a galactolipid:galactolipid galactosyltransferase which catalyses an interlipid exchange of galactose [6–8]:

MGDG + MGDG → DGDG + diacylglycerol (3)

During the course of envelope membrane preparation, the latter enzyme, by its action on MGDG molecules, induces the rapid formation of diacylglycerol and unnatural galactolipids such as tri- (tri-GDG) and tetra-galactosyldiacylglycerol (tetra-GDG):

MGDG + DGDG → tri-GDG + diacylglycerol (4)

In this communication, we demonstrate that the galactolipid:galactolipid galactosyltransferase is located on the outer surface of the outer membrane of the chloroplast envelope.

2. MATERIALS AND METHODS

2.1. *Chloroplast envelope purification*

Chloroplast envelope membranes were obtained from non-treated or thermolysin-treated intact spinach chloroplasts according to [9], except that the pH of all the different media used was adjusted to 8.0.

2.2. *Thermolysin treatment of isolated intact chloroplasts*

We used a non-penetrating proteolytic enzyme (thermolysin from *Bacillus thermoproteolyticus*, Worthington) to hydrolyse envelope polypeptides accessible on the outer surface of the outer envelope membrane [10]. The intact and purified chloroplasts (1 mg chlorophyll/ml; 4×10^8 chloroplasts/ml) were incubated for 1 h at 4°C in the following medium: 0.3 M sucrose; 10 mM tricine–NaOH (pH 7.8); 1 mM $CaCl_2$ and thermolysin (200 μg/ml). In order to terminate the reaction 10 mM EGTA was added. Control experiments (non-treated chloroplasts) were done in the same conditions except that the incubation medium contained at the same time both thermolysin and EGTA.

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Thermolysin-treated and non-treated, intact chloroplasts were then purified again through a Percoll gradient [9].

2.3. *Determination of envelope lipids*

Extraction, separation and quantification of envelope lipids were done according to [11].

2.4. *Lipid biosynthesis*

The assay for galactolipid synthesis was based on the extent of conversion of galactose from UDP-[^{14}C]galactose (water soluble) to several envelope membrane polar lipids (galactolipids). The complete reaction mixture contained: 10 mM tricine–NaOH buffer (pH 7.7); 1 mM UDP-[^{14}C]galactose (2 μCi/μmol); 1 mM $MgCl_2$ and enzyme (50 μg envelope protein) in a total volume of 0.2 ml. The labelled galactolipids were separated according to [11].

The assay for phosphatidic acid and diacylglycerol synthesis was based on the extent of conversion of *sn*-[^{14}C]glycerol 3-phosphate (water soluble) to several envelope lipids (lysophosphatidic acid, phosphatidic acid and diacylglycerol). The complete reaction mixture contained: 10 mM tricine–NaOH buffer (pH 8); 4 mM ATP; 0.2 mM CoASH; 1 mM *sn*-[^{14}C]glycerol 3-phosphate (5 μCi/μmol) and enzyme (20 μg of envelope protein + 0.4 mg of chloroplast extract protein) in a total volume of 0.2 ml. After 90 min incubation, 1 mM UDP-galactose was added. The labelled polar lipids were separated according to [11].

3. RESULTS

3.1. *Envelope lipid composition*

The polar lipid composition of envelope membranes from non-treated and thermolysin-treated intact spinach chloroplasts is shown in table 1. In good agreement with previous results [2], this table clearly indicates that envelope membranes from non-treated chloroplasts and prepared at pH 8.0 contained large amounts of diacylglycerol and non-negligible amounts of tri-GDG and tetra-GDG. On the contrary, envelope membranes from thermolysin-treated chloroplasts were devoid of diacylglycerol, tri-GDG and tetra-GDG. In addition, the amount of MGDG in envelope membranes from thermolysin-treated chloroplasts was much higher than that measured in envelope membranes from non-treated chloroplasts. Finally, DGDG, sulfolipid and phospholipids (such as phosphatidylcholine) were present in similar amounts in envelope membranes from both thermolysin- and non-treated chloroplasts.

These results demonstrate that treatment of intact spinach chloroplasts by thermolysin prevented the interlipid exchange of galactose during the course of envelope membrane preparation. Since thermolysin is a non-penetrant proteolytic enzyme which hydrolyzes only polypeptides located on the cytosolic surface of the chloroplast envelope [10], these results also demonstrate that the galactolipid:galactolipid galactosyltransferase is located on the outer surface of the outer membrane.

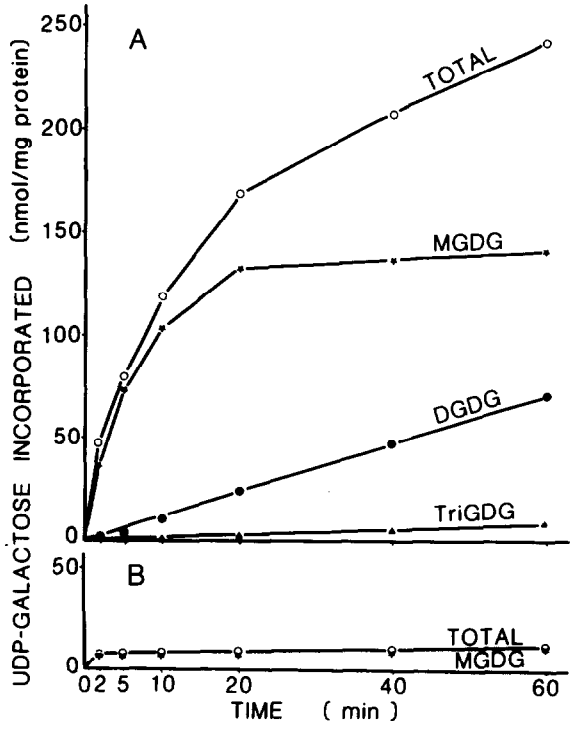

Fig.1. Galactolipid synthesis by envelope membranes isolated from non-treated (A) and thermolysin-treated (B) intact spinach chloroplasts. Incubation mixture and lipid extraction were as described under Materials and Methods. Lipids (labelled with [^{14}C]galactose from UDP-[^{14}C]galactose) were chromatographed in one dimension on silica-gel precoated TLC plates (Merck). Solvent system: chloroform/methanol/water (65:25:4, by vol.).

Table 1

Lipid composition of envelope membranes from non-treated and thermolysin-treated intact spinach chloroplasts

Lipids	Non-treated (μg fatty acids/mg protein)	(%)	Thermolysin-treated (μg fatty acids/mg protein)	(%)
MGDG	154	13.5	454	38
DGDG	381	33.5	354	30
TGDG	36	3	0	0
TTGDG	16	1.5	0	0
Sulfolipid	86	7.5	85	7
Phosphatidylcholine	172	15	160	13.5
Phosphatidylglycerol	102	9	108	9
Phosphatidylinositol	34	3	25	2
Phosphatidylethanolamine	0	0	0	0
Diacylglycerol	155	13.5	tr	<0.1
Total	1136	99,5	1185	99.5

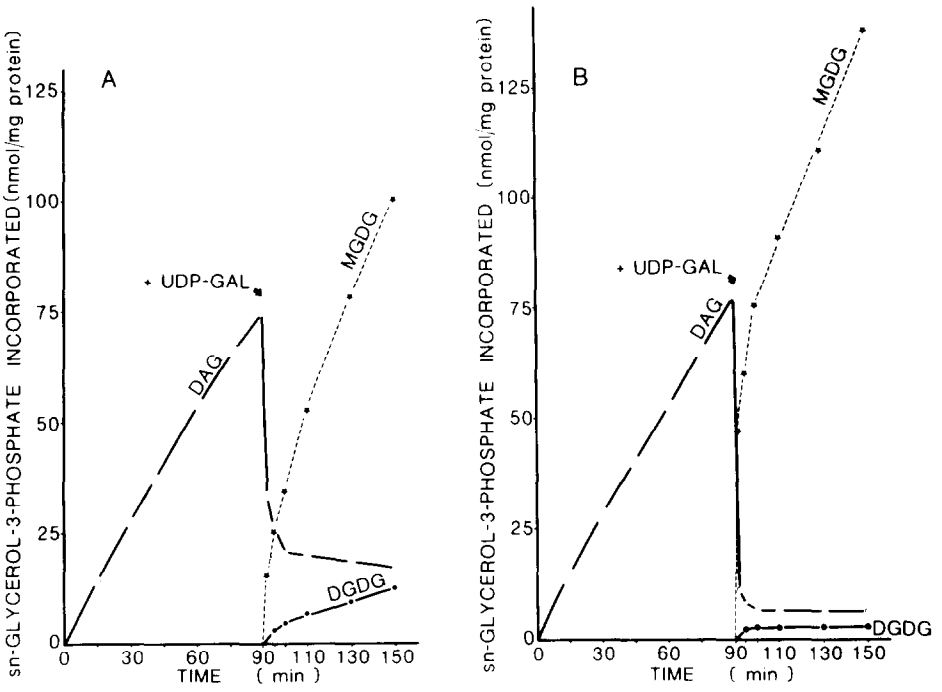

Fig.2. Diacylglycerol and MGDG synthesis by envelope membranes from non-treated (A) and thermolysin-treated (B) intact spinach chloroplasts. Incubation mixture and lipid extraction were as described under Materials and Methods. Lipids (labelled with *sn*-[^{14}C]glycerol from *sn*-[^{14}C]glycerol 3-phosphate) were chromatographed in one dimension on silica-gel precoated TLC plates (Merck). Solvent system: chloroform/methanol/water (65:25:4, by vol.). Only diacylglycerol and MGDG have been represented in this figure. As described in previous publications [11,12], incubation of envelope membranes and chloroplast extract (see Materials and Methods) with *sn*-glycerol 3-phosphate led to the formation of lysophosphatidic acid, phosphatidic acid and diacylglycerol.

3.2. *Lipid biosynthesis*

The incorporation of galactose from UDP-[^{14}C]galactose into envelope galactolipids is shown in fig.1. In the case of envelope membranes from non-treated chloroplasts (fig.1A) the synthesis of galactolipids and the kinetics of MGDG, DGDG, tri-GDG and tetra-GDG are in agreement with our previous observations [4]. On the contrary, in the case of envelope membranes from thermolysin-treated chloroplasts, the incorporation of galactose from UDP-[^{14}C]galactose into envelope galactolipids was almost completely inhibited (fig.1B). Such a result was predictable since envelope membranes from thermolysin-treated chloroplasts were practically devoid of diacylglycerol (table 1). However, such a situation could also reflect the destruction of the galactosylation enzymes during thermolysin treatment of intact chloroplasts. Consequently, in order to rule out this hypothesis, we have verified that diacylglycerol molecules actively synthesized by the envelope membranes from *sn*-[^{14}C]glycerol 3-phosphate (Kornberg–Pricer pathway) were rapidly incorporated into MGDG in the presence of UDP-galactose. Figure 2 shows that envelope membranes from non-treated and thermolysin-treated chloroplasts incorporated at the same rate *sn*-[^{14}C]glycerol 3-phosphate into membrane lipids and especially into diacylglycerol (see also [12]). In both cases, the addition of UDP-galactose to the incubation medium triggered a fast decrease in the amount of radioactivity incorporated into diacylglycerol which was accompanied simultaneously by a rapid synthesis of MGDG. It is interesting to note that the rate of disappearance of labelled diacylglycerol molecules in envelope membranes from thermolysin-treated chloroplasts (fig.2B) was much higher than that observed in envelope membranes from non-treated chloroplasts (fig. 2A). In fact, in the last case, the newly synthesized labelled diacylglycerol molecules were diluted by the large, pre-existing cold pool of diacylglycerol (table 1). Consequently, these results demonstrate that all the enzymes involved in the synthesis of MGDG (viz., acyl-CoA:*sn*-glycerol 3-phosphate acyltransferase, acyl-CoA:acyl-*sn*-glycerol 3-phosphate acyltransferase, acyl-CoA synthetase, phosphatidate phosphohydrolase and UDP-galactose:diacylglycerol galactosyltransferase) are protected from thermolysin digestion of intact chloroplasts.

4. DISCUSSION

The work reported here provides evidence that the galactolipid:galactolipid galactosyltransferase is located on the outer surface of the outer envelope membrane. As a matter of fact, treatment of intact spinach chloroplasts by a non-penetrating proteolytic enzyme (thermolysin) fully inhibits the interlipid exchange of galactose and this in turn prevents: (a) the formation of diacylglycerol at the expense of MGDG during the course of envelope membrane preparation; and (b) the incorporation of galactose from UDP-[^{14}C]galactose into MGDG.

One intriguing question raised by these observations is how the galactolipid:galactolipid galactosyltransferase becomes active during the purification of envelope membranes from intact chloroplasts. In contrast with previous reports [7], we have shown that the pH of the envelope isolation medium (from 7.0 to 9.0) was practically without effect on the lipid composition of the isolated envelope [13]. In addition, since isolated intact spinach chloroplasts are practically devoid of diacylglycerol [13], it is likely that the lipid composition of envelope membranes from thermolysin-treated chloroplasts represents the *in vivo* situation. However, during the course of gentle disruption of intact chloroplasts by hypotonic treatment, the outer and the inner envelope membranes fuse along their breaking edges [9]; this being so, it is very likely that the galactolipid:galactolipid galactosyltransferase has access to the inner membrane and triggers interlipid exchange of galactose at the expense of MGDG located on the inner membrane.

Finally, our results raise also the question of the physiological role of the galactolipid:galactolipid galactosyltransferase. This enzyme could be either a specific galactosidase involved in DGDG synthesis or an unspecific galactosidase. The fact that this enzyme: (a) is located on the outer face of the outer envelope membrane; (b) is probably a peripheral protein (see [10]); and (c) catalyses the synthesis of unnatural galactolipids (tri-DGDG and tetra-GDG) suggest, but do not prove, that this enzyme is probably not involved directly in DGDG synthesis. It is clear therefore that the enzyme responsible for DGDG synthesis still remains to be identified.

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