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Comparison of the kinetic properties of MGDG synthase in mixed micelles and in envelope membranes from spinach chloroplast

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Abstract We have applied the 'membrane partition' kinetic modelling approach proposed by Heirwegh et al. [(1988) Biochem. J. 254, 101–108] to MGDG synthase in isolated envelope vesicles. Comparison of the kinetic parameters obtained for MGDG synthase assayed in purified envelope membranes and in mixed-micelles demonstrates that the latter are relevant to the situation in envelope membranes and that MGDG synthase has a very high affinity for dilinoleoylglycerol. Our results provide additional evidence for the hypothesis that the high affinity of the envelope MGDG synthase for dilinoleoylglycerol could be responsible for the presence of C18 fatty acids at both the *sn*-1 and *sn*-2 position of the glycerol backbone in MGDG.

Key words: Chloroplast; Membrane; Mixed micelle; Monogalactosyldiacylglycerol; Diacylglycerol; Kinetic model

1. Introduction

MGDG (monogalactosyldiacylglycerol), the major plastid membrane polar lipid, is synthesized by a UDP-galactose: 1,2-diacylglycerol 3-β-D-galactosyltransferase (EC 2.4.1.46) or MGDG synthase, which transfers a galactose from a water-soluble donor, UDP-galactose (UDP-gal), to a hydrophobic acceptor molecule, diacylglycerol. We have recently investigated the kinetic mechanisms of the delipidated enzyme with respect to various molecular species of diacylglycerol [1]. These experiments were carried out in mixed micelles containing the partially purified enzyme [2], the substrate (diacylglycerol) and a detergent (CHAPS), according to the 'surface dilution' kinetic model proposed by Deems et al. [3]. Such experiments were designed to mimic the situation within the membrane in which the lipid constituents are constrained in the two-dimensional surface. The determination of the kinetic parameters of MGDG synthase towards diacylglycerol is difficult to perform in isolated envelope membranes because these membranes contain high levels of diacylglycerol [4]. Envelope diacylglycerols are produced within the membranes during the course of their purification owing to a galactolipid: galactolipid galactosyltransferase [5]. However, it is possible to prepare diacylglycerol-free envelope membranes by using thermolysin-treated chloroplasts for envelope purification: under these experimental conditions, the galactolipid: galactolipid galactosyltransferase (localized on the cytosolic side of the outer envelope membrane) is hydrolyzed and no diacylglycerol can be formed through this pathway [6]. Therefore, to provide further evidence for the physiological significance of the kinetic data obtained with mixed micelles, we decided to determine the kinetic parameters of MGDG synthase for diacylglycerol within envelope membranes prepared from thermolysin-treated intact chloroplasts.

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2. Material and methods

2.1. Materials

Pure synthetic PC molecular species (18:1/18:1 and 18:2/18:2) and phosphatidylglycerol (PG) were purchased from Sigma (L'Ile d'Abeau, France). Purity of the diacylglycerols used was monitored by thin layer chromatography (Silica gel 60, Merck) using petroleum ether/diethyl ether/acetic acid (80:20:1, v/v) as a solvent system. 1,2-Diacylglycerol was rapidly used to prevent isomerization into 1,3-diacylglycerol. Thermolysin (EC 3.4.24.4), a protease isolated from *Bacillus thermoproteolyticus*, was purchased from Boehringer (Meylan, France). CHAPS (3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propane sulfonate), a zwitterionic detergent, was obtained from Sigma. Unlabelled and ^{14}C-labelled (11.0 GBq/mmol) UDP-galactose were obtained, respectively, from Sigma and from New England Nuclear.

2.2. Purification of intact spinach chloroplasts

Chloroplasts were prepared from spinach (*Spinacia oleracea* L.) leaves and purified further by isopycnic centrifugation in Percoll gradients as described by Douce and Joyard [7]. Thermolysin-treated chloroplasts were obtained and purified as described by Dorne et al. [6].

2.3. Purification of envelope membranes from purified intact spinach chloroplasts

Envelope membranes were prepared from thermolysin-treated or non-treated intact chloroplasts using a step sucrose gradient as described by Douce and Joyard [7]. Envelope membranes (10 mg protein/ml) were stored, in liquid nitrogen, in 50 mM MOPS-NaOH, pH 7.8, and 1 mM DTT [8].

2.4. Partial purification of MGDG synthase

MGDG synthase was solubilized and partially purified from envelope membranes as described by Covès et al. [8] and Maréchal et al. [2]. This fraction was reasonably pure since, on average, a 30-fold purification (compared to solubilized envelope membranes) was obtained. On the other hand, the yield was low (less than 10% of the initial activity and only 0.1–0.2 mg protein). The lipid content of the fraction was below the limit of detection of the method and was therefore estimated to be lower than 0.1 mg/mg protein. Thus, the fractions containing the partially purified MGDG synthase activity used in this work were largely delipidated [1].

2.5. Assay of MGDG synthase

Assays with mixed micelles containing CHAPS (4.5 mM), PG (1.3 mM) and diacylglycerol (160 μM) were mostly performed as described by Maréchal et al. [1]. MGDG synthase activity was also assayed in isolated envelope vesicles. Since the purpose of our work was to determine the kinetic parameters for diacylglycerol, it was essential

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that the membranes used were devoid of endogenous diacylglycerol. This was achieved first by using envelope membranes prepared from thermolysin-treated chloroplasts. The traces of diacylglycerol which could remain in these envelope preparations were then converted into MGDG prior to the assay of MGDG synthase as follows. Envelope membranes (4 mg protein) were first incubated for 90 min in the following medium (final volume: 16 ml): 50 mM MOPS-NaOH (pH 7.8), 1 mM DTT, 250 mM KH_2PO_4/K_2HPO_4, 250 mM KCl and 600 μM UDP-gal. To remove both UDP-gal and UDP from the envelope suspension, 2.5 ml of the envelope suspension was loaded on top of a 0.6 M sucrose cushion (10 ml) containing 50 mM MOPS-NaOH (pH 7.8), 1 mM DTT, and centrifuged for 1 h at 250,000 × *g* (rotor SW 40; Sorvall). The membranes in the resulting pellet were resuspended in 6 ml of incubation medium containing 50 mM MOPS-NaOH (pH 7.8), 1 mM DTT, 250 mM KH_2PO_4/K_2HPO_4, 250 mM KCl. Various amounts of diacylglycerol dissolved in chloroform were introduced in glass tubes. After evaporation of the solvent under a stream of argon, 400 μl of diluted diacylglycerol-free envelope suspension was added. The tubes were vigorously mixed and kept for 1 h at 20°C. The reaction was then started by addition of 1 mM UDP-[^{14}C]gal (25 Bq/μmol). 120 μl aliquots were then taken after 1, 2 and 6 min incubation for lipid analyses and determination of the initial velocity of the reaction. The activity was expressed as μmol of galactose incorporated per h per mg protein. Experiments were reproduced at least three times.

2.6. Lipid determination

Glycerolipids were extracted from the different membrane fractions or medium according to Hajra [9]. The fatty acids from each sample were converted into fatty acid methyl esters and their contents were determined by gas chromatography, using C17:0 as a standard, as described by Douce et al. [10]. The concentrations of diacylglycerol molecular species prepared as described above were precisely determined according to the same procedure. They were first expressed in mixed micelles as molar values and as mol fractions, corresponding in our mixed micelles to [diacylglycerol]/([PG] + [CHAPS] + [diacylglycerol]), as described by Maréchal et al. [1], and in envelope vesicles to [diacylglycerol]/([envelope glycerolipids] + [diacylglycerol]).

2.7. Protein and chlorophyll determination

Protein concentration was determined according to Lowry et al. [11] using bovine serum albumin as a standard. In very dilute samples, proteins were first precipitated with 10% TCA, as described above. After centrifugation, the pellet was dried and the proteins determined with BCA reagent (Pierce). Chlorophyll concentration in chloroplast suspensions was measured according to Arnon [12].

3. Results

Envelope membranes from thermolysin-treated chloroplasts have a very low level of endogenous diacylglycerol [6]. The experimental procedure described in section 2 was designed to exclude most of the backgound due to the traces of diacylglycerol which could remain in the membrane. We therefore assayed MGDG synthase activity in such diacylglycerol-free envelope membranes supplied with exogenous diacylglycerol. Our previous results with mixed micelles [1] led us to use for these experiments the following diacylglycerol molecular species: dioleoylglycerol, which was representative of most of the diacylglycerol molecular species analyzed, and dilinoleoylglycerol which was the best substrate for the enzyme. In contrast to the data obtained in mixed micelles (Fig. 1A and C), rather complex double-reciprocal plots of the results were obtained with envelope vesicles for each diacylglycerol molecular species (Fig. 1B and D). At very low diacylglycerol concentrations, the velocity of the reaction was not dependent on diacylglycerol concentration and remained almost constant. Therefore, reciprocal plots of the data were almost parallel to the 1/[substrate] axis. In contrast, at high diacylglycerol concentrations, MGDG synthase was inhibited and the curve was therefore asymptotic to the $1/V$ axis. Plots of the data were linear only within a narrow range of diacylglycerol concentrations, which was dependent on (i) the nature of diacylglycerol used and (ii) the amount of envelope membrane in the incubation mixture. Kinetic constants were determined from the linear part of the curves (Fig. 1B and D). In this range of diacylglycerol concentrations, diacylglycerol mol fractions and molarities are proportional, allowing the determination of K_m expressed both in mol fractions and in μM (Table 1). The data presented in Fig. 1 lead to a series of observations. Firstly, the $V_{max,\,app}$ was much lower in isolated vesicles (≈ 0.5 μmol/h/mg protein) than in mixed micelles (10–12 μmol/h/mg protein). This was expected since mixed micelles contained partially purified MGDG synthase, i.e. there is more MGDG synthase molecules per surface unit

Table 1
Kinetic parameters of MGDG synthase in mixed micelles and in envelope vesicles

Diacylglycerol molecular species	Mixed micelles			Envelope membrane vesicles					
				Apparent Michaelis parameters (170 μM envelope lipids)			Extrapolated Michaelis parameters		
	K_m (mol fraction)	V_{max} (μmol galactose incorporated/h/mg protein)	V_{max}/K_m	K_m (mol fraction)	V_{max} (μmol galactose incorporated/h/mg protein)	V_{max}/K_m	K'_m (mol fraction)	V_{max} (μmol galactose incorporated/h/mg protein)	V_{max}/K'_m
18:1/18:1	0.028	11.1	399	0.1	0.5	5	0.033	0.5	15.15
18:2/18:2	0.0089	10.5	1176	0.0093	0.5	54	–	–	–

MGDG synthase activity was assayed in mixed micelles as described by Maréchal et al. [1] and in diacylglycerol-free envelope vesicles (see section 2). Each diacylglycerol molecular species is indicated by the two fatty acids esterifying, respectively, the *sn*-1 and *sn*-2 position of glycerol. Diacylglycerol concentration was expressed as mol fraction, i.e. [diacylglycerol]/([CHAPS] + [PG] + [diacylglycerol]) for mixed micelles, and [diacylglycerol]/([envelope glycerolipids] + [diacylglycerol]) for isolated envelope membranes. Double reciprocal plots of the results were drawn and used for determination of the kinetic parameters of the reaction (Fig. 1). The V_{max} and $K_{m,\,app}$ values were calculated from, respectively, the $1/V$ intercept and the 1/[substrate], expressed as mol fraction, intercept axes, as shown in Fig. 1. Extrapolated Michaelis parameters were determined from the following equation: $K_{m,\,app} = K'_m\,(1 + P \cdot N)$, for $N = 0$ and taking into account that both sides of the membrane leaflet could contain diacylglycerol, whereas the enzyme is probably only on one side of the membrane, as described in the text. –, not determined.

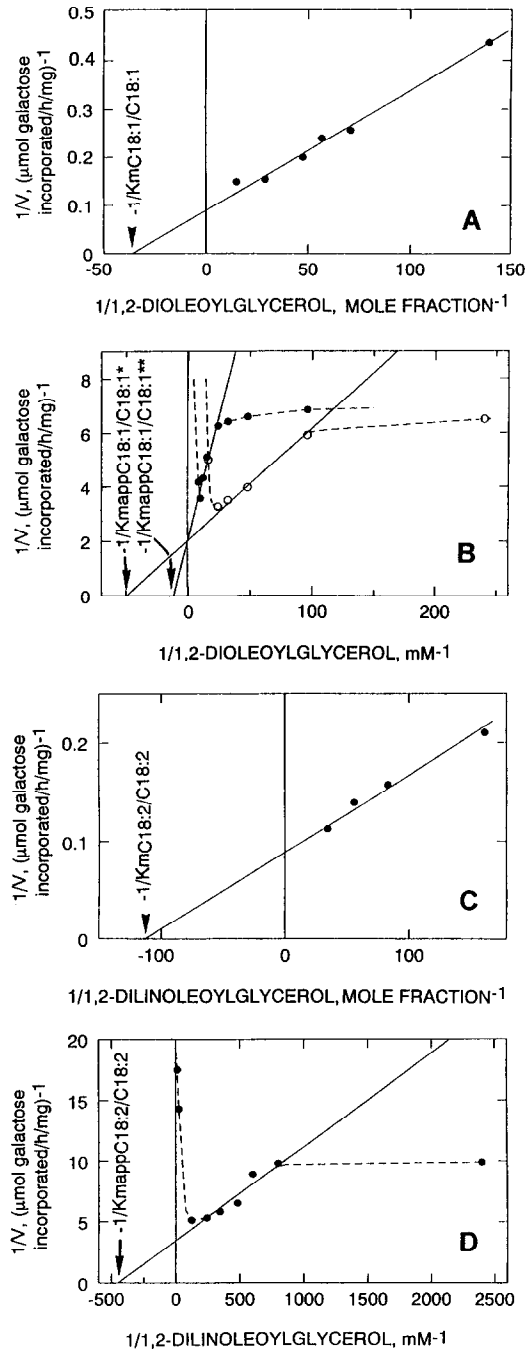

Fig. 1. Kinetic analyses of MGDG synthase activity in mixed micelles, as a function of diacylglycerol concentration, and in envelope membranes. MGDG synthase activity was determined with dioleoylglycerol (A and B) and dilinoleoylglycerol (C and D) as the varied substrate. Diacylglycerol concentration is expressed as mol fraction, [diacylglycerol]/([CHAPS] + [PG] + [diacylglycerol]) in mixed micelles, and in molar values for experiments with envelope membranes. MGDG synthase activity was assayed in mixed micelles as described by Maréchal et al. [1] and in diacylglycerol-free envelope vesicles as described in section 2. With dioleoylglycerol as the varied substrate, MGDG synthase activity was assayed in the presence of different amounts of envelope membranes: ○, 250 µg protein (K_m 18:1/18:1*); ●, 500 µg protein (K_m 18:1/18:1**) envelope proteins. With dilinoleoylglycerol as the varied substrate, MGDG synthase activity was assayed with envelope membranes corresponding to 250 µg protein. K_m values were determined by extrapolation of the linear part of the curves. They were shown to be dependent not only on the nature of the diacylglycerol used, as expected, but also on the amount of membrane added to the incubation mixture.
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in mixed micelles than in envelope vesicles. Secondly, like in mixed micelles, dilinoleoylglycerol was a much better substrate than dioleoylglycerol for MGDG synthase within the envelope membrane. For instance, the ratio of V_{max}/K_m values are 10 times higher for dilinoleoylglycerol than for dioleoylglycerol (Table 1). Since this ratio relates overall enzyme specificity for diacylglycerol [13], the specificity of the MGDG synthase for dilinoleoylglycerol is even more obvious in envelope membranes than in mixed micelles (Table 1). In addition, the K_m value for dilinoleoylglycerol was extremely low (2.2 µM or 0.0093 mol fraction) compared to the value obtained in mixed micelles. Fig. 1B also demonstrates that the apparent K_m values for diacylglycerol determined in envelope vesicles are dependent on the amount of membranes: the apparent K_m values determined for dioleoylglycerol were 20 and 90 µM (0.1 and 0.14 mol fraction) when the incubation mixture contained, respectively, 250 and 500 µg protein/ml.

4. Discussion

MGDG synthase is likely to be localized on the outer leaflet of the inner envelope membrane and therefore, most of the enzyme is probably localized on the inner leaflet of the envelope vesicles [7,14]. At very low diacylglycerol concentrations, it is possible that the substrate is present only in the outer leaflet of the vesicle, and therefore that only a minor part of the enzyme has access to the substrate, thus explaining that reciprocal plots of the data were almost parallel to the 1/[substrate] axis. In contrast, at high diacylglycerol concentrations, a strong inhibition by excess diacylgycerol was observed. Therefore, only a narrow range of diacylglycerol concentrations could be used for the kinetic analyses.

The purpose of this article was to question the relevance of the kinetic data obtained with mixed micelles to the situation in envelope membranes. We analyzed the data obtained with envelope membranes according to the 'membrane partition' kinetic modelling approach proposed by Heirwegh et al. [15,16] since all the six assumptions necessary for applying this model [16] were reasonably verified in our system. Indeed, (i) the envelope bilayer membrane can be represented as a quasi-bidimensional highly organized anisotropic fluid; (ii) all aqueous compartments can be regarded as a single aqueous phase, because of the structure of the envelope vesicles consisting of fused outer and inner envelope membranes [7,17] and containing a porine-like protein that allows the transfer of molecules of M_r lower than 10,000 [18]; (iii) diacylglycerol is a very hydrophobic molecule [19] and we observed that equilibration of the mixture of envelope vesicles with diacylglycerol, for 1 h at 20°C, was essential for reproducible results. Therefore, we can assume that, in our experiments, the system used was under equilibrium conditions, i.e. that exogenous diacylglycerol molecules added to envelope vesicles were totally inserted into the membrane. Under these conditions the interaction between MGDG synthase and its substrate proceeds only by lateral

diffusion, in agreement with the two-dimensional structure of the membrane. (iv) It is also assumed that the amount of MGDG synthase-bound diacylglycerol is negligible compared to the total amount of diacylglycerol. In support of this suggestion, we have demonstrated that the envelope MGDG synthase is only a very minor enzyme constituent of the inner envelope membrane [2]. (v) Moreover, in the range of diacylglycerol concentrations which are negligible compared to the total lipid amount present in the samples, diacylglycerol mol fractions and molarities are proportional and can be indifferently utilized to measure the kinetic parameters (see section 3 and Table 1). Finally, (vi) it is assumed that all steps of mass transport in the multiphasic systems are reversible and rapid compared to the rate of transformation of the substrate. Under these conditions, an increase in the amount of membrane necessarily implies an increase in the membrane surface, which, at constant molar substrate concentrations, results in a dilution of the surface concentration of the substrate. This simple consideration was used by Heirwegh et al. [15] to demonstrate that the apparent K_m values should increase linearly with the amount of membrane, whereas the V_{max} values should remain constant. This was exactly the result we obtained (Fig. 1B), in good agreement with kinetic studies with membrane-bound enzymes such as sialidase from neuronal membranes [20]. The effective dissociation constant for dioleoylglycerol can be determined by plotting apparent K_m values (in mol fractions) vs. envelope lipid concentration (N, see below), according to the following equation:

$$K_{m,\,app} = K'_m\,(1 + P \cdot N)$$

where P (diacylglycerol partition coefficient) is the mol fraction of ligand included in vesicles per mol fraction of ligand in aqueous compartement, and N is the mol of lipids included in vesicles per mol of solvent (N is proportionnal to the bulk lipid concentration: N = [lipid]/[solvent], with [solvent] estimated as equal to water molarity, i.e. 55.6 M, see [15,16]). Under these conditions the extrapolated K_m value for dioleoylglycerol was 0.067 mol fraction. If we now consider that the actual diacylglycerol surface concentration available to MGDG synthase is two times lower because (i) diacylglycerol would be present in each leaflet of the bilayer structure of the membrane (in contrast to a micelle, which does not present such a structure) and (ii) that in a vesicle MGDG synthase is present only on one side of the membrane [14], the effective dissociation constant should be around 0.033, which is in the order of magnitude of the value obtained with mixed micelles, i.e. 0.028 (Table 1). Moreover, the diacylglycerol partition coefficient deduced is P = 7070, in agreement with the very high hydrophobicity of this substrate. Therefore, we can conclude that the situation in mixed micelles is relevant to the situation in envelope membranes. Indeed, the data presented in this study confirm our previous experiments with mixed micelles and provide further evidence (i) that the envelope MGDG synthase does not have the same affinity for all diacylglycerol molecular species, and (ii) that dilinoleoylglycerol is the best substrate. Our results also provide additional evidence for the hypothesis [1] that only the high affinity of the envelope MGDG synthase for dilinoleoylglycerol is responsible for the presence of C18 fatty acids at both *sn*-1 and *sn*-2 positions of the glycerol backbone in MGDG, since this molecular species is not formed at high rates in the inner envelope membrane, in contrast to diacylglycerol molecular species containing 18:1 and 16:0 fatty acids, respectively, at the *sn*-1 and *sn*-2 positions of the glycerol backbone [21,22].

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