Direct desaturation of intact galactolipids by a desaturase solubilized from spinach (*Spinacia oleracea*) chloroplast envelopes*

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In plants, polyenoic fatty acids are synthesized by desaturase enzymes which use acyl groups of membrane lipids as substrates. To provide direct ‘*in vitro*’ evidence for this reaction, we solubilized envelope membranes from spinach (*Spinacia oleracea*) chloroplasts with Triton X-100 to release a membrane-bound *n*–6 desaturase. In the presence of oxygen and reduced ferredoxin, the solubilized enzyme desaturated a variety of substrates, such as free oleic acid, free erucic acid, 1-oleoyl-*sn*-glycerol 3-phosphate and the three galactolipids 1-oleoyl-2-(7′-*cis*-hexadecenoyl)-3-β-D-galactopyranosyl-*sn*-glycerol, 1,2-dioleoyl-3-β-D-galactopyranosyl-*sn*-glycerol and

the ether analogue 1,2-di-(9′-*cis*-octadecenyl)-3-β-D-galactopyranosyl-*sn*-glycerol. The *in vitro* desaturation of these exogenously added complex lipids with ester- and ether-linked substrate chains is unambiguous evidence for lipid-linked desaturation. The enzyme measures the insertion of the new double bond from the methyl end and the existing (*n*–9)-*cis*-double bond of an appropriate acyl or alkyl chain. The distal part of the substrate group, normally the carboxy end of a fatty acyl residue, is of less importance and, in particular, its activation in thioester form is not required.

INTRODUCTION

Membrane lipids of plant cells contain high proportions of polyunsaturated fatty acids. Biochemical and genetic work has shown that, subsequent to the formation of monoenoic acids in chloroplasts, separate desaturase systems in chloroplast and microsomal (endoplasmic reticulum, ER) membranes catalyse the sequential insertion of the second and third double bonds, resulting in the accumulation of dienoic and trienoic acids, such as linoleic and linolenic acid [1–3]. In both compartments oxygen is the terminal acceptor for electrons derived from the prospective double-bond position of the acyl group and from additional electron donors which have recently been identified as ferredoxin (Fd) in chloroplasts [4] and cytochrome *b₅* in microsomes [5,6]. In contrast with animal desaturases, which use acyl thioester derivatives of CoA as substrates [7,8], plant enzymes produce polyenoic acids by introducing double bonds into acyl chains, which as ester groups are part of polar membrane lipids. In chloroplasts the preferred substrates for dienoic and trienoic acid biosynthesis are acyl chains of monogalactosyldiacylglycerol (MGD), whereas in ER membranes phosphatidylcholine is desaturated at the highest rates.

This lipid-linked desaturation was originally deduced from detailed analyses of *in vivo* labelling experiments using various radioactive tracers. In the course of such experiments the acyl groups of phospho- and glyco-lipids became increasingly more unsaturated [9,10], whereas acyl-CoA [11] and acyl-acyl carrier protein [12] pools are not accepted by desaturases for introduction of second or third double bonds. M.s. analysis of ¹³C-labelled glycolipids has suggested that, in photosynthetically active cyanobacteria also, membrane lipids are the actual desaturase substrates [13]. On the other hand, for a long time it was difficult to present *in vitro* evidence for this desaturation of intact lipids; however, in the meantime, various subcellular

systems, including intact [14–19] and ruptured chloroplasts [4,20] or envelope [21] and microsomal membrane preparations (summarized in [3]) have been described in which desaturation is most easily explained as involving the acyl groups of added or *in situ*-generated glyco- and phospho-lipids.

To provide additional and direct evidence for this lipid-linked desaturation, we solubilized a desaturase from chloroplast envelope membranes and established an ‘*in vitro*’ system which operates with both enzymes and substrates in solubilized form. Under these conditions the desaturase from chloroplasts was active with preformed galactolipid substrates carrying *cis*-monoene alkyl chains in ester or ether linkage. The introduction of a second double bond into these substrates is unequivocal evidence for a direct lipid-linked desaturation.

EXPERIMENTAL

Biochemicals

Radiochemicals were purchased from Amersham and CEA, with a specific radioactivity of 50.8–56 μCi/μmol. Fd, NADP⁺:ferredoxin oxidoreductase (FNR), acyl-CoA synthetase and phospholipase C (from *Bacillus cereus*) were from Sigma, whereas catalase and lipase (from *Rhizopus*) were obtained from Boehringer. Triton X-100 (10% solution from Pierce) was peroxide-free. Acyl-acyl carrier protein: *sn*-glycerol-3-phosphate acyltransferase with a specific activity of 3.2 μmol/min per mg was a gift from Dr. M. Frentzen.

Plant material and general methods

Spinach (*Spinacia oleracea* L., cv. Subito) was grown hydroponically in a phytotron, and only young leaves were used for isolation of chloroplasts via Percoll gradients as described previously [19]. Extraction of lipids, separation of individual

Abbreviations used: Fd, ferredoxin; FNR, ferredoxin:NADP⁺ oxidoreductase; MGD, monogalactosyldiacylglycerol; ER, endoplasmic reticulum; C_{18:1}/C_{16:1} MGD, 1-oleoyl-2-hexadecenoyl-3-β-D-galactopyranosyl-*sn*-glycerol; C_{18:1} LPA, 1-[1-¹⁴C]oleoyl-*sn*-glycerol 3-phosphate; C_{18:1}/C_{18:1} MGD-ether, 1,2-di-(9′-*cis*-octadecenyl)-3-β-D-galactopyranosyl-*sn*-glycerol; C_{18:1}/C_{18:1} MGD-ester, 1,2-di-[1-¹⁴C]oleoyl-3-β-D-galactopyranosyl-*sn*-glycerol.

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components, preparation of methyl esters, analysis of the positional distribution of fatty acids in lipids using *Rhizopus* lipase, hydrogenation of lipids with Pd/C as catalyst and chlorophyll determination in 80 % acetone were as described previously [4,17,19,21]. Membrane proteins solubilized with Triton X-100 were measured by the method of Bradford [22], with BSA (from Sigma), dissolved in the same Triton X-100 solution, as standard.

Isolation and solubilization of envelope membranes

Intact chloroplasts (10–14 mg of chlorophyll) in isolation buffer [40 mM Tricine/KOH (pH 8.0)/300 mM sorbitol] from 50–70 g of spinach leaves were sedimented at 3000 *g* for 2 min. The supernatant fluid was removed and the pellet was diluted with 10 ml of shock buffer [10 mM Tris/KOH (pH 8.5)/5 mM MgCl₂] for osmotic breakage of chloroplasts. The resultant mixture was supplemented with 50000 units of catalase and placed on a stepped sucrose gradient (in a 14 ml tube) formed by two layers (2 ml each) of sucrose solutions (0.6 and 1 M) in shock buffer. After centrifugation for 10 min at 200000 *g*, the yellow envelope membranes were recovered from the 0.6/1 M sucrose interface. This fraction (~1 ml) was diluted with 1 ml of shock buffer containing 10000 units of catalase and centrifuged for 5 min at 200000 *g*. The sediment was resuspended in 500 μ l of solubilization buffer [20 mM Hepes/KOH (pH 7.6)/20 % glycerol] and adjusted with Triton X-100 to a detergent/protein ratio of 4:1 (w/w). After centrifugation for 30 min at 200000 *g* the supernatant fraction contained solubilized envelope proteins.

Assay for oleate desaturation

The activity of oleate desaturase in the solubilized envelope proteins (1–10 μ g of protein) was measured in a total volume of 100 μ l, with the final concentrations (given in parentheses) of the following components: spinach Fd (44 μ g), FNR (20 units), NADPH (5 mM), catalase (2000 units), MgCl₂ (5 mM), Triton X-100 (1 mM), Mops/KOH (50 mM, pH 7.5) and 28.4 μ M [1-¹⁴C]oleic acid (5.5 kBq). After 20 min incubation at room temperature the reaction was stopped with 10 μ l of 90 % acetic acid containing 10 % Triton X-100 and 5 μ l of trichloroacetic acid (3 M). The precipitated proteins were sedimented by a short centrifugation (1 min, 1000 *g*), and an aliquot (50 μ l) of the supernatant fraction containing the non-esterified fatty acids was directly injected for h.p.l.c. analysis of the desaturation products.

Radio-h.p.l.c.

Separation of non-esterified fatty acids was performed isocratically on a column (4.6 mm $\times$ 125 mm) of ODS-hypersil RP18 (3 μ m particle size) with methanol/acetonitrile/water/acetic acid (900:100:130:1, by vol) at a flow rate of 1 ml/min. For other lipids the solvent was methanol/acetonitrile (9:1, v/v), with different proportions of water: 15 % for fatty acid methyl esters; 5 % for MGD (ester form); and 2 % for MGD (ether analogue). Radioactivity in the effluent was continuously monitored by a flow-through scintillation detector system [19].

Preparation of labelled substrates

For the synthesis of ¹⁴C-labelled 1-oleoyl-2-hexadecenoyl-3- β -D-galactopyranosyl-*sn*-glycerol (C_{18:1}/C_{16:1} MGD) intact chloroplasts (2 mg/ml chlorophyll) were preincubated with KCN (100 μ M) for 60 min to inhibit the *n*-6- and *n*-3-desaturases

[23]. The subsequent labelling of MGD from 10 μ Ci of [1-¹⁴C]-acetate was carried out in 2.5 ml of incubation medium as described previously [4] for 90 min in the light with chloroplasts (500 μ g of chlorophyll) and the components required for MGD synthesis (UDP-galactose and glycerol 3-phosphate). After extraction of lipids, MGD was purified by t.l.c. for subsequent separation of molecular species by h.p.l.c. C_{18:1}/C_{16:1} MGD was collected (0.64 μ Ci), freed from solvent by argon evaporation and dispersed in 1 mM Triton X-100 (10000 d.p.m./ μ l) by ultrasonication.

1-[1-¹⁴C]Oleoyl-*sn*-glycerol 3-phosphate (C_{18:1} LPA) was synthesized enzymically from labelled oleic acid in a volume of 1 ml with the following components (final concentrations or quantities in parentheses): 175 μ M [1-¹⁴C]oleic acid (10 μ Ci), 4 mM *sn*-glycerol 3-phosphate, 0.2 % BSA, 10 mM ATP, 5 mM CoA, 2.5 mM dithioerythritol, 10 mM MgCl₂, 0.1 % Triton, 0.2 M Mops/KOH, pH 7.5, 0.4 units of acyl-CoA synthetase (from Sigma) and 7.2 μ g of acyl-acyl carrier protein: *sn*-glycerol-3-phosphate acyltransferase. After 3 h of incubation the lipids were extracted by phase partitioning [24], the solvents evaporated with argon and the non-esterified fatty acids removed by extraction with 2 ml of light petroleum (b.p. 40–60 °C). The residual C_{18:1} LPA was dispersed in 10 mM Tricine/KOH, pH 8.0.

The tritiated 1,2-di-(9'-*cis*-octadecenyl)-3- β -D-galactopyranosyl-*sn*-glycerol (C_{18:1}/C_{18:1} MGD-ether) from a previous synthesis [25,26] was purified from degradation products by h.p.l.c. as described above. For incubation of the MGD-ether with solubilized envelope proteins, it was dispersed in 1 mM Triton X-100 (50000 d.p.m./ μ l).

1,2-Di[1-¹⁴C]oleoyl-3- β -D-galactopyranosyl-*sn*-glycerol (C_{18:1}/C_{18:1} MGD-ester) was synthesized with thermolysin-treated chloroplast envelopes from 1,2-di-[1-¹⁴C]oleoyl-*sn*-glycerol and UDP-galactose [27]. A 5 μ Ci portion of di-[1-¹⁴C]oleoyl phosphatidylcholine was evaporated under argon and dispersed by ultrasonication in 1.25 ml of buffer (30 mM H₃BO₃/10 mM Tris/HCl, pH 7.5). The dispersed lipid was incubated with 50 units of phospholipase C (from *Bacillus cereus*) and 2 ml of diethyl ether for 30 min at room temperature. The diethyl ether was removed and the aqueous layer was extracted twice with 2 ml of diethyl ether. The combined diethyl ether extract was washed with 2 ml of water and the solvent removed by evaporation under argon. The dried diacylglycerol was redispersed by ultrasonication in 100 μ l of 20 mM Tricine/KOH, pH 7.5, containing 125 nmol of lysophosphatidylcholine.

The dispersed diacylglycerol was incubated for 1 h with a suspension (100 μ l) of envelope membranes (200 μ g of protein) in a final volume of 250 μ l of 0.2 M NaCl and 0.8 mM UDP-galactose. After extraction with chloroform/methanol the lipids were purified by t.l.c. and the molecular species of MGD separated by h.p.l.c. as described above. The C_{18:1}/C_{18:1} MGD (1.3 μ Ci) was dispersed in 1 mM Triton X-100 (10000 d.p.m./ μ l).

RESULTS AND DISCUSSION

In previous experiments with chloroplasts, the highest oleate desaturase activity was measured in envelope membranes [21]. We therefore considered this membrane system as a useful source for isolating the membrane-bound desaturase. As a first step we established conditions for solubilization and measuring maximal rates of this activity. As a consequence of the results shown in Figure 1(c), we used a Triton X-100/protein ratio of 4 for solubilization of envelope membrane pellets in the absence of salt, followed by centrifugation (30 min at 200000 *g*), after which the activity is recovered in the supernatant fraction. The

Figure 1 Optimization of the oleate desaturase assay

Envelope membranes were solubilized at a detergent/protein ratio of 4:1 (w/w) and centrifuged at 200000 g for 20 min. From the supernatant fraction 5 μg of protein were incubated in a total volume of 100 μl for 20 min at pH 7.5 with the electron-transport components (NADPH, FNR and Fd; see the Experimental section) and increasing concentrations of free $[1-^{14}\text{C}]$ oleic acid (**a**). The desaturation products were directly analysed as non-esterified fatty acids by h.p.l.c. Variation of activity with assay pH (**b**) was measured at 28 μM $[1-^{14}\text{C}]$ oleic acid. Desaturase solubilization with increasing detergent concentration is shown in (**c**). Aliquots of envelope membranes (150 μg of protein) were incubated in the same volume (300 μl) in the presence of increasing concentrations of Triton X-100 and subjected to ultracentrifugation. Aliquots of the supernatant fractions containing identical quantities of protein (5 μg) were assayed under standard conditions for total activity (right ordinate). Solubilized protein increased continuously and was highest at the highest detergent concentration.

Figure 2 Acceptance of different substrates by the solubilized desaturase

(a) 1- $[1-^{14}\text{C}]$ oleoyl-*sn*-glycerol 3-phosphate ($\text{C}_{18:1}$ -LPA); (b) non-esterified $[1-^{14}\text{C}]$ oleic acid ($\text{C}_{18:1}$ NEFA); (c) non-esterified $[1-^{14}\text{C}]$ erucic acid ($\text{C}_{22:1}$ NEFA). Each substrate (0.05 μCi) was incubated with solubilized envelope proteins for 2 h under standard conditions. The desaturation products were analysed either in the form of methyl esters prepared by transesterification (**a**) or directly as non-esterified fatty acids (**b** and **c**).

solubilized enzyme is most active in 1 mM Triton X-100 between pH 7.0 and 8.0 (Figure 1b) and saturated with 28 μM non-esterified oleic acid as substrate (Figure 1a) in the presence of NADPH, FNR, ferredoxin and catalase. Under these conditions rates of 1.5 nmol/min per mg of protein are routinely measured, whereas without catalase hardly any activity can be detected. This could be attributed to the inhibitory effect of H_2O_2 formed by autoxidation of reduced Fd [28]. The reaction rate was constant up to 20 min (corresponding to about 10% desaturation of the substrate) and proportional to protein up to 10 μg per assay (results not shown).

The use of free oleic acid as a simple substrate had been suggested by experiments with envelope membranes in which, in

Figure 3 Desaturation of ^{14}C -labelled 1-oleoyl-2-(7'-*cis*-hexadecenoyl)-3- β -D-galactopyranosyl-*sn*-glycerol ($\text{C}_{18:1}/\text{C}_{16:1}$ MGD-ester)

Solubilized envelope proteins (10 μg) were incubated under standard conditions with 0.025 μCi of substrate (**a**) for 2 h. One experiment was used to analyse the desaturation products in the form of molecular species of the intact lipid (**b**). In the other, desaturation products were subjected to a positional analysis of fatty acid distribution by lipase hydrolysis. The fatty acids from the *sn*-1 (**c**) and *sn*-2 position (**d**) were analysed as methyl esters.

addition to MGD, other lipids, including non-esterified fatty acids, had been desaturated to various extents [21]. In confirmation of these results, Figure 2 shows that, apart from oleic acid, 1-oleoyl-*sn*-glycerol 3-phosphate too is readily accepted, and even erucic acid (13-*cis*- $\text{C}_{22:1}$) is desaturated to a

Figure 4 Desaturation of $C_{18:1}/C_{18:1}$ MGD-ester

Solubilized envelope proteins (19 μ g) were incubated for 2 h with 0.025 μ Ci of substrate (a). One experiment was used to analyse the desaturation products in the form of molecular species of the intact lipid (b). In the other, desaturation products were subjected to a positional analysis of fatty acid distribution by lipase hydrolysis. The fatty acids from the *sn*-1 (c) and *sn*-2 (d) position were analysed as methyl esters.

dienoic acid which most likely carries the two *cis* double bonds in the $\Delta^{13,15}$ (or $n-9,6$) positions. This result demonstrates that the desaturase has a low specificity towards the C-terminal part of the acyl substrate and identifies the position of the second double bond by measuring its distance both from the methyl end of the substrate and the existing double bond. Therefore, this desaturase meets the criteria of an ($n-6$)-desaturase operating with ($n-9$) substrates [29].

The availability of the desaturase in solubilized form provided the possibility for *in vitro* incubations with solubilized lipids of complex structure. For this purpose we prepared three labelled galactolipids having a common backbone of 3-*O*- β -D-galactopyranosyl-*sn*-glycerol, but differing in the acyl or alkyl substituents in the *sn*-1 and *sn*-2 positions. The first substrate ($C_{18:1}/C_{16:1}$ MGD; Figure 3) was prepared by incubating isolated chloroplasts with [14 C]acetate, *sn*-glycerol-3-phosphate and UDP-galactose in the presence of 100 μ M KCN. Owing to the selective inhibition of the monoene desaturase activity [23], the molecular species of MGD carrying oleic acid at C-1 and ($n-9$)-hexadecenoic acid at C-2 accumulated and was isolated in sufficient quantity by preparative h.p.l.c. (Figure 3a). The galactolipid with this fatty acid pairing serves as a representative of prokaryotic substrates which are formed within the chloroplasts. The second substrate ($C_{18:1}/C_{18:1}$ MGD; Figure 4) represents the eukaryotic lipids in chloroplasts formed from diacylglycerol backbones imported from the ER and carrying

two C_{18} fatty acids with various extents of unsaturation [1,30]. The labelled dioleoyl-MGD was formed from 1,2-di-[1- 14 C]-oleoyl-*sn*-glycerol released by phospholipase C from accordingly labelled phosphatidylcholine and incubated with isolated chloroplast envelopes in the presence of UDP-galactose. The appropriate molecular species was purified from the total MGD by preparative h.p.l.c. (Figure 4a). The third substrate (Figure 5) is the 9',10'-tritiated ether analogue of the second compound, i.e., 1,2-di-*O*-(9'-*cis*-octadecenyl)-3-*O*- β -D-galactopyranosyl-*sn*-glycerol. It was available from previous studies [25,26], but it had to be purified from degradation products by h.p.l.c.

The incubation of $C_{18:1}/C_{16:1}$ MGD, followed by analysis of the desaturation products as molecular species of intact lipids, revealed two additional peaks (Figure 3b) with shorter retention times corresponding to products with additional double bonds. A positional analysis of the fatty acids in the unfractionated galactolipid recovered from this incubation shows that the acyl residues in both positions at the glycerol backbone are desaturated to a comparable extent: in the C-1 position oleic acid is desaturated to linoleic acid and at C-2 hexadecenoic is converted into hexadecadienoic acid (Figures 3c and 3d). The possible combinations of these fatty acids result in products with a total of three or four double bonds corresponding to the two additional peaks resolved by h.p.l.c. (Figure 3b). *In vivo* these species are intermediates during the desaturation of the *de novo*-formed $C_{18:1}/C_{16:0}$ MGD, leading to the finally accumulating $C_{18:3}/C_{16:3}$ MGD [17].

The corresponding experiment with $C_{18:1}/C_{18:1}$ MGD results in a similar desaturation pattern (Figure 4b) with two product peaks representing different molecular species with additional double bonds. The positional analysis of fatty acids (Figures 4c and 4d) demonstrates that, in this substrate also, both positions are desaturated to the same extent, i.e. in both positions oleic is converted into linoleic acid. The demonstration that eukaryotic chloroplast lipids with dioleoyl backbones are readily desaturated by the envelope desaturase supports the previously held notion [31] that it is not necessarily a di-linoleoyl backbone which is imported from the ER, as is sometimes stated. The desaturation of 7-*cis*- $C_{16:1}$, 9-*cis*- $C_{18:1}$ and 13-*cis*- $C_{22:1}$ shows that the ($n-6$)-desaturase accepts ($n-9$) substrates of different chain length and that acyl groups of lipids with 16 and 18 carbon atoms in both positions of the glycerol backbone are desaturated with equal efficiency and without discrimination between pro- and eukaryotic substrates. This conclusion assumes that, in chloroplasts, the introduction of the second double bond into different substrates at different positions is catalysed by a single *cis*-($n-6$)-desaturase. Evidence for this was obtained from genetic experiments which have shown that a single nuclear mutation results in the inhibition of both hexadecenoic and oleic acid desaturation in different chloroplast lipids [32]. At the same time the results shown in Figures 3 and 4 demonstrate that our solubilization procedure does not allow the recovery of activity required for the conversion of dienoic into trienoic acyl residues.

The desaturation of the dioctadecenyl ether substrate is shown in Figure 5. After incubation, two product peaks were observed (Figure 5b), which, owing to their chromatographic behaviour and by analogy to the ester substrates, are considered as desaturation products with a total of three and four double bonds respectively. This is supported by catalytic hydrogenation, which converted these products into the fully saturated dioctadecyl ether having a higher retention time (Figure 5c). The desaturation of the ether substrate is direct evidence for lipid-linked desaturation in chloroplasts, since even a transient exchange as possible with acyl groups of ester substrates can be excluded with ether-linked octadecenyl residues.

Figure 5 Desaturation of $C_{18:1}/C_{18:1}$ MGD-ether

Solubilized envelope proteins (19 μ g) were incubated for 2 h with 0.25 μ Ci of tritiated substrate (a) under standard conditions. Desaturation products were separated as molecular species before (b) or after (c) hydrogenation.

Ester and ether galactolipids can be separated by reversed-phase h.p.l.c., since ester compounds, owing to their additional carbonyl oxygen atoms, are eluted earlier than ethers. We therefore incubated a mixture of the di- $C_{18:1}$ -ester and the di- $C_{18:1}$ -ether galactolipid and separated the products formed in this experiment by a single h.p.l.c. run. The faster eluted molecular species of the ester substrate showed the same desaturation pattern as the separately following ether products (results not shown).

Again, this result shows a relaxed selectivity of the desaturase towards the distal part of its substrate, which is in obvious contrast with the data obtained from labelling of intact chloroplasts. With these organelles it has repeatedly been shown

[14,17–19] that 1-acyl-glycerol 3-phosphate, 1,2-diacylglycerol 3-phosphate and diacylglycerol contain mainly palmitic and oleic acid and only small proportions of dienoic and trienoic acids, which, on the other hand, are readily detected in MGD. This discrepancy between the unselectivity of the desaturase observed *in vitro* and its selectivity *in vivo* may be for different reasons. The desaturase may, in fact, have a preference for certain lipids, the demonstration of which will require a detailed kinetic analysis with solubilized substrates. On the other hand, the pools of the above-mentioned intermediates *in vivo* are very small when compared with the polar lipids that are the finally accumulating products. The excess of these undoubtedly good substrates competes for binding to the desaturase, and this, together with a possible substrate channelling [33], may contribute to the apparent selectivity of the desaturase observed *in vivo*. The difference between plant and animal desaturases may be greater in the mode of substrate binding than in the actual desaturation reaction mechanism. The large distance between the carboxy group at C-1 and the position of double bonds at C-6, C-9, C-12, or C-15 will cancel any influence of the thioester group, which, in animal desaturases also, may not be required for the actual desaturation. The only means of enzymic differentiation between methylene groups in this remote region may be by distance measurement from reference points [29] that include the existing double bond and the methyl end or the carboxy group in its various derivative forms. Further purification of the desaturase enzymes and the possibility of incubations with different substrates in solubilized form should help in answering these questions.

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