Substrate specificities of the enzymes of the oleate desaturase system from photosynthetic tissue

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In the microsomal fraction from young pea (*Pisum sativum* L.) leaves, the oleoyl moieties from oleoyl-CoA are principally transferred to the *sn*-2 position of phosphatidylcholine by oleoyl-CoA:1-acyl-lysophosphatidylcholine acyltransferase. The major product of this acyl transfer is 1-palmitoyl(stearoyl)-2-oleoyl phosphatidylcholine. The 1-palmitoyl(stearoyl)-2-oleoyl phosphatidylcholine is subsequently converted into 1-palmitoyl(stearoyl)-2-linoleoyl phosphatidylcholine by the oleate desaturase complex without equilibrating with the bulk membrane phosphatidylcholine pool. Hence, both the acyl transfer to phosphatidylcholine and the subsequent desaturation of oleoyl moieties occur on the *sn*-2 position of phosphatidylcholine, and there is also a functional coupling of the acyltransferase and oleate desaturase.

It is now generally accepted that, in both photosynthetic tissues (e.g. leaves) and in storage tissues (e.g. developing seeds), desaturation of oleate to linoleate occurs in membranes which can be isolated in the microsomal fraction (Roughan & Slack, 1982). Oleate is originally synthesized in the plastids and is subsequently exported to the cytosol as oleoyl-CoA for further metabolism (Stumpf *et al.*, 1980). It was originally proposed that $C_{18:1}$ -CoA is directly desaturated to yield $C_{18:2}$ -CoA (Vijay & Stumpf, 1972; Trémolières *et al.*, 1980). Most later studies indicate, however, that the actual desaturase substrate is a $C_{18:1}$ -containing phosphatidylcholine, which yields a $C_{18:2}$ -containing phosphatidylcholine as a product (Slack *et al.*, 1976, 1979; Stymne & Appelqvist, 1978; Stumpf *et al.*, 1980; Stymne & Glad, 1981; Stymne *et al.*, 1983; Stobart *et al.*, 1983; Murphy *et al.*, 1983, 1984a,b,c). More recently it has been proposed that, in microsomes (microsomal fractions) from photosynthetic tissue, the desaturase substrate is synthesized from $C_{18:1}$ -CoA and a 1-acyl lysophosphatidylcholine via an acyltransferase that is functionally coupled to the desaturase complex (Murphy *et al.*, 1984a,b,c). In the present study we

have examined this reaction sequence in more detail and determined the identities of the molecular species of phosphatidylcholine involved in both acyl transfer and desaturation.

Materials and methods

Microsomes were isolated from the leaves of 8-day-old pea (*Pisum sativum*, cultivar 'kleine Rheinländerin') seedlings by the Mg^{2+} -precipitation technique (Diesperger *et al.*, 1974; Murphy *et al.*, 1984b). The microsomal pellets, containing about 1 mg of protein, were routinely incubated with 0.1 μ Ci of [$1-^{14}C$]oleoyl-CoA (60 μ Ci/ μ mol, Amersham International) in a medium (1 ml) consisting of 10 mM- $MgCl_2$, 1 mM-NADH, 0.3 mM-CoASH and 4 mM-ATP. Details of the methods used for incubation, extraction and purification of lipids and their analysis by t.l.c. and g.l.c. are given elsewhere (Murphy *et al.*, 1983, 1984a,b,c).

Distribution of acyl moieties at the *sn*-1 and *sn*-2 positions of phosphatidylcholine was determined as follows. Microsomal phosphatidylcholine, isolated by t.l.c., was hydrolysed with purified phospholipase A_2 from rattlesnake (*Crotalus durissus terrificus*) venom. (Boehringer) (Robertson & Lands, 1962). The products of hydrolysis, i.e. 1-acyl lysophosphatidylcholine and fatty acids, liberated from the *sn*-2 position of phosphatidylcholine, were isolated by t.l.c., converted into methyl esters,

Abbreviations used: $C_{x:y}$ phosphatidylcholine, $C_{x:y}$ -fatty-acid-containing phosphatidylcholine; $C_{x:y}$ -CoA, $C_{x:y}$ -fatty-acyl-CoA.

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and analysed by radio-g.l.c. employing the methods detailed previously (Murphy *et al.*, 1983, 1984a,b,c).

In order to determine the molecular species of phosphatidylcholine, the microsomal phosphatidylcholine isolated by t.l.c., was completely hydrolysed with phospholipase C from *Bacillus cereus* (Boehringer) (Hanahan & Vercamer, 1954). The product of hydrolysis, i.e. 1,2-diacylglycerols, was acetylated (Renkonen, 1966) and the resulting diacylglycerol acetates were resolved into various molecular species according to degree of unsaturation by t.l.c. on silica-gel G containing 10% (w/w) AgNO_3 with chloroform/methanol (99:1, v/v) as solvent. The individual molecular species were transmethylated and the resulting methyl esters were analysed by radio-g.l.c. as described elsewhere (Murphy *et al.*, 1983, 1984a,b,c).

Results and discussion

Coupling of acyl transfer with desaturation

A time course of the changes in specific radioactivity of the $\text{C}_{18:1}$ phosphatidylcholine and $\text{C}_{18:2}$ phosphatidylcholine formed after incubation of pea leaf microsomes with $[1\text{-}^{14}\text{C}]\text{C}_{18:1}\text{-CoA}$ is shown in Fig. 1. The specific radioactivity of $\text{C}_{18:1}$ phosphatidylcholine rose rapidly over the first 20 min of the incubation and reached a plateau thereafter. The kinetics of $\text{C}_{18:1}$ phosphatidylcholine labelling over the initial 60 min were essentially the same whether or not NADH was present in the incubations, despite the complete lack of desaturation in the absence of NADH. At later times there was a slight fall in the specific radioactivity of $\text{C}_{18:1}$ phosphatidylcholine in the absence of NADH, whereas in the presence of NADH the specific radioactivity of $\text{C}_{18:1}$ phosphatidylcholine remained essentially constant. In the presence of NADH, the formation of $\text{C}_{18:2}$ phosphatidylcholine was dependent on the presence of $\text{C}_{18:1}$ phosphatidylcholine and reached a constant rate at the same time as the attainment of a constant specific radioactivity of $\text{C}_{18:2}$ phosphatidylcholine was not reflected in a concomitant fall in that of $\text{C}_{18:1}$ phosphatidylcholine. This is in agreement with previous reports (Murphy *et al.*, 1984b,c) which proposed that the establishment of an equilibrium between the reactive $\text{C}_{18:1}\text{-CoA}$ and $\text{C}_{18:1}$ -phosphatidylcholine pools led to the maintenance of an essentially constant pool size of newly synthesized $\text{C}_{18:1}$ phosphatidylcholine during the incubations.

No formation of radioactive $\text{C}_{18:2}$ phosphatidylcholine was observed in the absence of NADH (Fig. 1). The addition of NADH after 40 min resulted in the accumulation of radioactivity in $\text{C}_{18:2}$ phosphatidylcholine at a similar rate to that

Fig. 1. Time course of changes in specific radioactivity of $\text{C}_{18:1}$ phosphatidylcholine ($\circ$, $\bullet$) and $\text{C}_{18:2}$ phosphatidylcholine ($\square$, $\blacksquare$) on incubation of pea leaf microsomes with $[1\text{-}^{14}\text{C}]\text{oleoyl-CoA}$.

Microsomal fractions (1 mg of protein), isolated from 8-day-old pea leaves, were incubated by shaking at 22°C with $0.1 \mu\text{Ci}$ of $[1\text{-}^{14}\text{C}]\text{oleoyl-CoA}$ ($60 \mu\text{Ci}/\mu\text{mol}$) in 1 ml of potassium phosphate buffer, pH 7.2, containing 1% bovine serum albumin, 10 mM- MgCl_2 , 0.3 mM- CoASH and 4 mM-ATP. Microsomal phosphatidylcholine was isolated from the reaction products by t.l.c., transmethylated, and the methyl esters analysed by radio-g.l.c. as described in the Materials and methods section. $\circ$, $\text{C}_{18:1}$ phosphatidylcholine and $\square$, $\text{C}_{18:2}$ phosphatidylcholine in the presence of NADH (1 mM); $\bullet$, $\text{C}_{18:1}$ phosphatidylcholine and $\blacksquare$, $\text{C}_{18:2}$ phosphatidylcholine in the absence of NADH.

observed if NADH were added at the start of the incubation (results not shown). However, in the former case there was very little lag time between addition of NADH and appearance of the desaturation product. This was probably due to the availability of a pool of newly synthesized $\text{C}_{18:1}$ phosphatidylcholine to act as a substrate for the desaturase. The complete blocking of desaturase activity by the omission of NADH from the incubation medium also resulted in an increase in $\text{C}_{18:1}\text{-CoA}$ thiosterase activity, as measured by the accumulation of oleic acid (results not shown).

Identity of phospholipid substrates

In previous papers we demonstrated that 1-acyl lysophosphatidylcholine was a more efficient acceptor of $\text{C}_{18:1}$ moieties from $\text{C}_{18:1}\text{-CoA}$ than either diacyl phosphatidylcholine or glycerol-3-phosphate (Murphy *et al.*, 1984b,c). This implied that a 1-acyl-2- $\text{C}_{18:1}$ phosphatidylcholine was likely to be the immediate substrate for the oleate desaturase. This possibility has been investigated by following a time course of the distribution of radioactivity among the 1-acyl and 2-acyl moieties

of phosphatidylcholine after incubation of pea leaf microsomes with [1-¹⁴C]C_{18:1}-CoA, as shown in Table 1. Under desaturating conditions, over 80% of the C_{18:1} radioactivity incorporated into phosphatidylcholine was found in the 2-acyl moieties after a 15 min incubation. Longer-term incubations resulted in some decrease in this proportion to 57% after 35 min. C_{18:2} radioactivity was also mainly concentrated in the 2-acyl moieties and, although some C_{18:2} radioactivity was also found in the 1-acyl moieties, the specific radioactivity of C_{18:2} at the *sn*-2 position was always 3–5-fold higher than at the *sn*-1 position. The appearance of labelled C_{18:1} or C_{18:2} moieties in the *sn*-1 position during the later stages of incubation is probably due to acyl transfer from the *sn*-2 position via deacylation/reacylation and/or acyl exchange (Stymne & Glad, 1981; Stymne *et al.*, 1983; Stobart *et al.*, 1983) rather than direct acyl transfer of the

C_{18:1} moieties to the *sn*-1 position and subsequent desaturation. The latter reaction cannot be positively ruled out at this stage, but if it does occur, it is a relatively minor process compared with the desaturation of 2-C_{18:1} phosphatidylcholine. These data provide strong evidence that the *sn*-2 position of phosphatidylcholine is the site of attachment of C_{18:1} moieties from C_{18:1}-CoA and that the 2-acyl moieties of the phosphatidylcholine so formed are the sites of C_{18:1} desaturation in pea leaf microsomes.

The identity of the phospholipid substrates of the microsomal acyltransferase and desaturase may be deduced from molecular-species analysis of the phosphatidylcholine products after incubation with [1-¹⁴C]C_{18:1}-CoA, as shown in Table 2.

The major molecular species of microsomal phosphatidylcholine that became radioactively labelled were: species containing one double bond,

Table 1. Positional distribution of radioactive C_{18:1} and C_{18:2} moieties in microsomal phosphatidylcholine after incubation with [1-¹⁴C]oleoyl-CoA

Microsomal fraction (1 mg of protein), isolated from 8-day-old pea leaves, was incubated by shaking at 22°C with 0.1 µCi of [1-¹⁴C]oleoyl-CoA (60 µCi/µmol) in 1 ml of potassium phosphate buffer, pH 7.2, containing 1% bovine serum albumin, 10 mM-MgCl₂, 0.3 mM-CoASH, 4 mM-ATP and 1 mM-NADH. Microsomal phosphatidylcholine was isolated from the reaction products by t.l.c., hydrolysed with phospholipase A₂ (from *Crotalus durissus terrificus* venom), and the resulting products, i.e. 1-acyl lysophosphatidylcholine and fatty acids liberated from the *sn*-2 position of phosphatidylcholine, were isolated by t.l.c., converted into methyl esters and analysed by radio-g.l.c. as described in the Materials and methods section.

Incubation time (min)	Position Fatty acid	Distribution (%) of radioactivity in phosphatidylcholine (specific radioactivity, nCi/nmol)			
		<i>sn</i> -1		<i>sn</i> -2	
		C _{18:1}	C _{18:2}	C _{18:1}	C _{18:2}
15		18.4 (1.15)	0.5 (0.01)	80.5 (2.82)	0.6 (0.05)
25		26.1 (2.19)	1.0 (0.02)	70.4 (2.71)	2.5 (0.06)
35		35.7 (2.22)	2.3 (0.03)	57.1 (2.13)	4.9 (0.10)

Table 2. Distribution of radioactivity in molecular species of microsomal phosphatidylcholine after incubation with [1-¹⁴C]oleoyl-CoA

Microsomal fraction, isolated from 8-day-old pea leaves, was incubated with [1-¹⁴C]oleoyl-CoA under the conditions given in Table 1 with or without added NADH (1 mM). Microsomal phosphatidylcholine was isolated from the reaction products by t.l.c. and hydrolysed with phospholipase C (*Bacillus cereus*); the resulting 1,2-diacylglycerols were acetylated, and the diacylglycerol acetates fractionated by argentation t.l.c. into molecular species according to degree of unsaturation; the individual species were transmethyalted and the methyl esters analysed by radio-g.l.c. as described in the Materials and methods section. Abbreviation used: tr, traces.

Distribution of radioactivity [%]							
NADH	Species 1 (C _{16:0} , C _{18:0} /C _{18:1})		Species 2 (C _{16:0} , C _{18:0} /C _{18:2} + C _{18:1} /C _{18:1})			Species 3 (C _{16:0} , C _{18:0} /C _{18:3} + C _{18:1} /C _{18:2})	
	% of total	C _{18:1} (%)	% of total	C _{18:1} (%)	C _{18:2} (%)	% of total	C _{18:1} (%)
+	48	100	18	52	48	26	100
-	48	100	14	100	tr	31	100

Scheme 1. Microsomal oleate metabolism

the $C_{16:0}$, $C_{18:0}/C_{18:1}$ combination; species containing two double bonds, that is a mixture of $C_{16:0}$, $C_{18:0}/C_{18:2}$ and $C_{18:1}/C_{18:1}$ combinations; and the species having three double bonds, which was a mixture of $C_{16:0}$, $C_{18:0}/C_{18:3}$ and $C_{18:1}/C_{18:2}$ combinations. Under both desaturating and non-desaturating conditions, about one-half of the total $C_{18:1}$ moieties transferred from $C_{18:1}$ -CoA to phosphatidylcholine was found in the $C_{16:0}$, $C_{18:0}/C_{18:1}$ species; in this species the saturated acyl moieties were found to constitute about 80% $C_{16:0}$ and 20% $C_{18:0}$. Under desaturating conditions, labelled $C_{18:2}$ moieties were detected only in species 2, which has the $C_{16:0}$, $C_{18:0}/C_{18:2}$ combination; also in this species the saturated acyl moieties consisted of approx. 80% $C_{16:0}$ and 20% $C_{18:0}$. Obviously the molecular species of phosphatidylcholine containing the $C_{16:0}$, $C_{18:0}/C_{18:1}$ combination is the major substrate for the oleoyl phosphatidylcholine desaturase, which yields the species containing the $C_{16:0}$, $C_{18:0}/C_{18:2}$ combination as the major product.

The conclusions from these results are summarized in Scheme 1.

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