SUB-CELLULAR LOCALIZATION OF THE OLEYL-CoA DESATURASE ACTIVITY IN PEA LEAVES

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1. Introduction

Oleyl-CoA desaturase is a key enzyme in the biosynthetic pathway of linoleic and linolenic acids [1,2] which are 'essential fatty acids' for the vertebrates, being formed only in plant tissues. Up to now this enzyme has been evidenced only in four microsomal fractions: from developing seeds of safflower *(Carthamus tinctorius)* [3], from *Neurospora crassa* [4], from potato tuber slices [1] and *Chlorella vulgaris* [5]. Recently, Tremolieres and Mazliak [2] and, Slack and Roughan [6] have suggested a possible cooperation between the microsomes and the plastids of plant cells in the biosynthesis of α-linolenic acid, the first desaturation (oleic acid into linoleic acid) occurring in the microsomes and the second one (linoleic acid into linolenic acid) occurring in the plastids. Slack and Roughan [6] also suggested from in vivo labelling experiments that phosphatidylcholine plays a role in the transport of linoleic acid from the microsomes to the plastids. Thus, it appeared important to localize the oleyl-CoA desaturase in the cells of plant leaves and to determine the molecular type of the lipid into which the newly produced linoleic acid was incorporated, in order to see whether or not this acid was incorporated into the eventual carrier, phosphatidylcholine.

2. Materials and methods

2.1. *Materials*

Pisum sativum (cv. Alaska) seedlings were grown in short day conditions (8 h of white light) at 22°C.

2.2. *Preparation of sub-cellular fractions*

20 g of young (six-day old) leaves were ground gently in a mortar in the following aqueous medium: sucrose 0.4 M, bovine serum albumin (BSA) 0.1%, KCl 10 mM, $MgCl_2$ 1 mM, cysteine-HCl 3 mM, Tris-HCl 0.1 M, pH 7.4.

The homogenate was filtered through 2 sheets of miracloth and the filtrate centrifuged at 500 *g* for 5 min; the first pellet was discarded and the supernatant centrifuged at 3000 *g* for 15 min; this second pellet was designated as 'crude plastid fraction' or '3000 *g* fraction'. This fraction was purified according to Tuquet [7] in a discontinuous sucrose gradient consisting of 10 ml of 1.5 M sucrose, 10 ml of 1 M sucrose, 10 ml of 0.75 M sucrose and 10 ml of 0.4 M sucrose containing the resuspended crude plastid pellet. After centrifugation at 1000 *g* for 10 min, purified intact plastids (class I according to Spencer and Unt [8]) were located by means of phase contrast microscopy just above the 1.5 M sucrose layer; disrupted chloroplasts (class II) were located in the bottom of the 0.75 M sucrose layer. The good physiological state of class I plastids was ascertained with an oxygen electrode by assaying the control of photosynthetic oxygen evolution induced by ADP, according to West and Wiskich [9].

The supernatant of the '3000 *g*' fraction was centrifuged at 25 000 *g* for 15 min. The pellet obtained designated as '25 000 *g* fraction' contained (as revealed by electron microscopy) mitochondria, fragments of plastids and some vesicles coated with ribosomes. The supernatant fraction was centrifuged at 100 000 *g* for 60 min. The pellet ('100 000 *g* fraction') contained mainly 'smooth' or 'rough' (coated with

ribosomes) microsomal vesicles; this fraction was slightly contaminated by fragments of photosynthetic lamellae (as shown by some chlorophyll content).

2.3. *Oleyl-CoA desaturase assay*

Crude or purified fractions were suspended in the isolation medium and gently homogenized in a Potter apparatus with a Teflon pestle; 0.2 ml of the suspension (1 to 1.5 mg of protein as determined according to Lowry et al. [10] were mixed with 1 μmol of NADH, 10 mg of BSA, 2 mg of $MgCl_2$ and 8 nmol of [1-^{14}C]oleyl-CoA in 0.1 M potassium phosphate buffer, pH 7.2 (final volume : 1 ml) according to Ben Abdelkader et al. [1]. Oleyl-CoA was prepared from Avocado mesocarp tissue according to Gaillard and Stumpf [11].

2.4. *Thin-layer chromatographic (t.l.c.) analysis of lipids*

This was performed according to Lepage [12] and Mangold [13]. The radioactivity of the spots was detected either by autoradiography or by scanning (Panax apparatus).

2.5. *Gas-liquid chromatographic analysis*

Lipids from the spots of t.l.c. or the incubation medium were saponified with 5 ml of 0.5 M methanolic NaOH for 15 min at 65°C; then the fatty acids were methylated by 2 ml of a 14% solution of boron trifluoride in methanol for 5 min at 65°C; the fatty acid methyl esters were extracted in 10 ml pentane. Gas-liquid radiochromatography was performed with a Barber-Colman, Nuclear Chicago (series 5000) apparatus on a column of butane-diol-succinate (20% on hexamethyl disilanized chromosorb W 60–80 mesh, at 190°C; the column was 3 m long and 6 mm in diameter). The flow rate of the carrier gas (argon) was 40 ml/min.

2.6. *Chlorophyll content*

Chlorophyll content was determined according to Arnon [14].

3. Results

Table 1 shows the protein, chlorophyll and fatty acid composition of the sub-cellular fraction '3000 *g*', '25 000 *g*', and '100 000 *g*', respectively. The last fraction contained little chlorophyll, and $C_{16:1\Delta3}$ *trans*, and had also the lowest percentage of α-linolenic acid.

Table 2 shows that the highest specific activity of oleyl-CoA desaturase was found in the '100 000 *g*'

Table 1
Biochemical properties of subcellular fractions from young pea leaves (20g)

(A) Protein, pigment and lipid distribution
(% of total amount in the membranes)

Fractions	Protein	Chlorophylls	Lipids
'3000 *g*'	61	67	47
'25 000 *g*'	22	27	26
'100 000 *g*'	17	6	27

(B) Fatty acid composition
(% of total fatty acids)

Fractions	$C_{16:0}$	$C_{16:1}$ *cis*	*trans*	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
'3000 *g*'	16	0.7	0.7	2.5	6	10	64
'25 000 *g*'	19	1	tr.	4	4	34	42
'100 000 *g*'	22	tr.	–	6	27	24	20

tr. = traces

Table 2
Comparison of 'in vitro' oleyl-CoA desaturase activity and 'in vivo' desaturation of oleic acid in subcellular fractions

Fraction	In vivo % of oleic acid desaturation	In vitro specific activity (nmoles of linoleate formed/mg protein in 30 min)
'3000 *g*'	26	0.02
Purified plastids	–	0.00
'25 000 *g*'	53	0.19
'100 000 *g*'	73	0.40
Supernatant	1	0.00

The 'in vivo' desaturation was measured by feeding ammonium oleate to the whole leaf for 6 h and then isolating the sub-cellular fractions as described in methods.

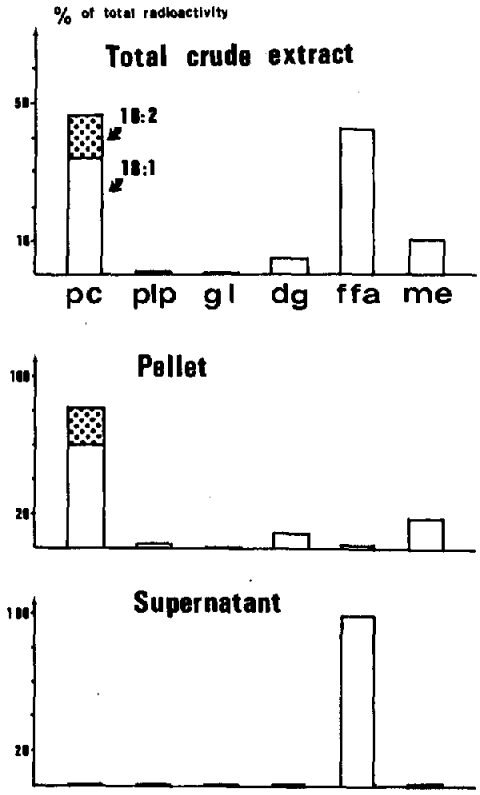

Fig.1. Incorporation of oleic acid ($C_{18:1}$) and newly produced linoleic acid ($C_{18:2}$) by the microsomal fraction from young pea leaves. The 'classes' of lipids were separated as described in the methods. PC, phosphatidylcholine; PLP, other phospholipids; GL, galactolipids; DG, diglycerides; FFA, free fatty acids; ME, unidentified lipid with the same R_f as fatty acid methylesters. Open columns, radioactivity in oleic acid; Dotted columns, radioactivity in linoleic acid.

or 'microsomal' fraction in which it was found to be higher than in the 'crude plastid fraction'. When the plastid fraction was purified, the oleyl-CoA desaturase activity became undetectable. On the other hand, the distribution of the oleyl-CoA desaturase activity follows very closely the sub-cellular pattern of 'in vivo' oleic acid desaturation by the whole leaf.

The microsomal oleyl-CoA desaturase activity follows the 'in vivo' synthesis of linoleic acid as well, during the development of the leaves. These two activities reach their highest levels, even in very young leaves, one or two days before the dramatic increase of α-linolenic acid content, that took place about 7 days after the beginning of germination, as shown by Tremolieres [15].

Fig.1 shows that, if oleate can be incorporated into phosphatidylcholine, diglycerides and free fatty acids, about all the linoleic acid formed by desaturation is incorporated into phosphatidylcholine.

If microsomes were pelleted after incubation with oleyl-CoA (100 000 *g* for 60 min) all the labelled phosphatidylcholine, diglycerides and fatty acid methyl esters were found in the pellet (and, in this case, all the linoleic acid formed was recovered in the phosphatidyl-choline of the pellet). The supernatant contained all of the free fatty acids. This was probably due to a lipase activity in our system.

It is interesting to notice that no incorporation of labelled fatty acids into other phospholipids and galactolipids could be detected. Thus, pea leaf microsomes show a great specificity for the acylation reaction.

4. Discussion

Our results clearly show that the major part, if not all, of the oleyl-CoA desaturase activity is located in microsomes of young pea leaves.

It is difficult to prove unequivocally, by negative findings, that no oleyl-CoA desaturase activity is present in the plastids. Nevertheless, this study and all previous work [2,6] showed that isolated plastids

are not able to synthesize de novo linolenic acid from acetate or from CO_2 (the studies of Kannangara and Stumpf [16] show an elongation of $C_{16:3}$ into $C_{18:3}$ but no de novo synthesis). These results support the hypothesis of a cooperation between plastids and microsomes in the biosynthesis of α-linolenic acid in green leaves (and the participation of phosphatidyl choline in this cooperation, cannot be excluded).

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