Solubilisation of oleoyl-CoA thioesterase, oleoyl-CoA:phosphatidylcholine acyltransferase and oleoyl phosphatidylcholine desaturase

The oleate desaturase system of pea leaf microsomes

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Received 1 September 1983

Membrane-bound enzymes involved in oleate metabolism in microsomes from pea (*Pisum sativum* L.) leaves were solubilised using detergents, such as *n*-octyl glucoside, Triton X-100, digitonin or cholate. The detergents were found to be inhibitory to oleoyl-CoA thioesterase, oleoyl-CoA:phosphatidylcholine acyltransferase and oleoyl phosphatidylcholine desaturase. Detergent removal by dialysis resulted in the restoration of activity of both the solubilised oleoyl-CoA thioesterase and oleoyl-CoA:phosphatidylcholine acyltransferase. The putative components of the oleoyl phosphatidylcholine desaturase system were also partially solubilised.

Microsome *Oleoyl-CoA thioesterase* *Oleoyl-CoA:phosphatidylcholine acyltransferase* *Oleoyl phosphatidylcholine desaturase*

1. INTRODUCTION

The biosynthesis of oleic acid in photosynthetic plant tissues is mediated by soluble enzymes in the chloroplast [1,2]. In such species as pea, cucumber and broad bean the oleate is then transferred to the cytosol where it is further metabolised on membrane-bound enzymes which are recovered in the microsomal fraction. We have recently reported the following enzymes from microsomes of young pea leaves: 18:1-CoA synthetase, 18:1-CoA thioesterase, 18:1-CoA:PC acyltransferase, and 18:1-PC desaturase [3], which are involved in the conversion of oleic acid to 18:2-PC. There is now growing evidence from tissues, such as potato tubers [4] and fungi such as *Tetrahymena pyriformis* [5,6], that there is a cytochrome b_5-mediated electron transport chain linked to oleate desaturation. This is similar to the electron transport chain of stearoyl-CoA desaturase found in animal tissues, such as rat and chicken livers [7–9].

In order to isolate and characterise the PC-dependent microsomal oleate desaturase of pea leaves it is necessary to study the effects of detergents on the activity and the extent of solubilisation of all the enzymes involved in

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Abbreviations: CoA, coenzyme A; PC, phosphatidylcholines; FA, unesterified fatty acids; 18:1, oleoyl moieties; 18:2, linoleoyl moieties

Published by Elsevier Science Publishers B.V.

metabolism of 18:1-CoA to 18:2-PC. This is because 18:1-CoA is currently the most suitable substrate for studies on oleate desaturation. We here report on the effects of detergents upon 18:1-CoA thioesterase, 18:1-CoA:PC acyltransferase and various putative components of the 18:1-PC desaturase system and on their partial solubilisation.

2. MATERIALS AND METHODS

Methods used for isolation of the microsomes from the leaves of 8-day old pea (*Pisum sativum*, cultivar Kleine Rheinländerin) seedlings, incubation, lipid extraction and analysis by thin-layer chromatography and gas chromatography have been described in detail [3].

Pea leaf microsomes containing about 1 mg protein were routinely incubated for 1 h with 0.1 μCi [1-^{14}C]oleoyl-CoA (56 mCi/mmol, Amersham-Buchler) in an incubation medium (1 ml), which consisted of 0.1 M potassium phosphate buffer (pH 7.2), 1% bovine serum albumin (fatty acid-free), 10 mM $MgCl_2$, 1 mM NADH, 0.3 mM CoA, 4 mM ATP and 2000 units catalase. Some incubations were carried out in the presence of varying amounts of detergents, such as *n*-octyl glucoside and Triton X-100. In each case, the activities of various enzymes were assessed by analysis of the radioactive reaction products [3]. The proportions of labelled unesterified fatty acid and phosphatidylcholine formed were considered as measures of the activities of 18:1-CoA thioesterase and 18:1-CoA:PC acyltransferase, respectively. The percentage of labelled linoleoyl moieties formed was considered to reflect the activity of 18:1-PC desaturase.

All steps for the solubilisation of microsomal proteins with detergents were carried out at 4°C. Freshly prepared pea leaf microsomes containing about 1 mg protein were suspended in 1-ml incubation medium containing various amounts of *n*-octyl glucoside, Triton X-100, digitonin or cholate and shaken gently for 2 h. The unsolubilised membranes were sedimented by centrifugation at 100000 × *g* for 2 h, separated from the supernatant and resuspended in the incubation medium (1 ml). The supernatant and pellet were each dialyzed against 10 mM potassium phosphate buffer (pH 7.2) for either 18 h or 43 h and the dialysis residues were assayed for enzymic activities as described.

For subcellular distribution studies, protoplasts were prepared from pea leaves by a modification of the method in [10]. Leaf slices were incubated in a digestion medium containing 0.5 M sorbitol, 1 mM $CaCl_2$, 5 mM 4-morpholineethanesulphonic acid, 0.5% bovine serum albumin, 3% cellulase and 0.5% macerozyme. Chloroplast and cytosol fractions were obtained by rapid centrifugation through a silicone oil layer [10]. The soluble and membrane-bound chloroplast and cytosol activities were further fractionated by centrifugation at 30000 × *g* to yield fractions enriched, respectively, in chloroplast stroma, thylakoids, soluble cytosol and microbodies; e.g., microsomes. The purity of the various fractions was assessed by means of enzymatic markers as in [10]. This technique yields a chloroplast fraction with less than 3% contamination by such extra-chloroplastic markers as phosphoenolpyruvate carboxylase and isocitrate dehydrogenase. The non-chloroplast fraction contained less than 12% of the total activity of the chloroplast markers; i.e., chlorophyll and NADP-linked glyceraldehyde-3-phosphate dehydrogenase. Each fraction was incubated with [1-^{14}C]oleoyl-CoA and the radioactive reaction products were analyzed, as described above, in order to assess enzyme activities.

3. RESULTS AND DISCUSSION

Subcellular fractionation studies (not shown) on pea leaf protoplasts revealed that over 90% of the total oleoyl-CoA:phosphatidylcholine acyltransferase activity was associated with the non-chloroplast membrane fraction. In contrast, 20–30% of the total long-chain acyl-CoA thioesterase activity was non-chloroplastic. Both of these figures are corrected for organellar cross-contamination as measured by marker enzymes.

The effects of detergents on the metabolism of [1-^{14}C]-18:1-CoA by pea leaf microsomes are shown in table 1. Both *n*-octyl glucoside and Triton X-100 inhibited 18:1-CoA:PC acyltransferase and 18:1-PC desaturase with increasing concentration of the detergent in the incubation mixture. The activity of 18:1-CoA thioesterase was relatively little affected by

Table 1

Effect of detergents on metabolism of [1-^{14}C]oleoyl-CoA by pea leaf microsomes

Detergent (conc. % w/v)		Radioactive 18:2 (% of total ^{14}C-labelled fatty acids)	Distribution of radioactivity in reaction products (% of total)			
			FA	PC	Acyl-CoA	Others[a]
None		32.0	10.1	21.8	58.0	10.1
n-Octyl glucoside	(0.15)	29.3	6.5	24.1	66.0	3.4
	(0.60)	11.9	5.6	26.6	65.0	2.8
	(1.50)	0.0	7.7	7.0	84.0	1.3
Triton X-100	(0.01)	30.0	10.2	41.4	40.0	8.4
	(0.10)	10.5	3.4	11.6	83.0	2.0
None		16.6	10.3	38.8	43.0	7.9
Triton X-100	(0.01)	20.1	12.2	30.6	49.0	8.2
	(0.10)	1.1	18.0	6.8	69.0	6.2

[a] Other minor products detected include diacylglycerols, triacylglycerols, lysophosphatidylcholines and phosphatidylethanolamines

Microsomes were isolated from leaves of 8-day old pea seedlings, incubated with [1-^{14}C]18:1-CoA in the presence of detergents, and the products were analyzed as described in section 2

detergent treatments under the conditions used. Inhibition of oleate desaturation by detergents has been observed earlier in microsomes from potato tubers [11].

Since the detergents were found to inhibit 18:1-CoA:PC acyltransferase and 18:1-PC desaturase (table 1), the assay of these enzymes in solubilised and non-solubilised microsomal fractions does not reveal the extent of their solubilisation if detergents are present in the assay mixture. Therefore, in one set of experiments (table 2) the solubilised and non-solubilised fractions, obtained from pea leaf microsomes by treatment with either 0.5% (w/v) cholate or 1.5% (w/v) *n*-octyl glucoside, were dialyzed for different periods in order to remove the detergents. Subsequently, the dialysis residues were assayed for enzymic activities.

The results, given in table 2, show that the activity of 18:1-CoA:PC acyltransferase and that of 18:1-CoA thioesterase in the non-solubilised fractions were affected to a minor extent by dialysis. However, a strong enhancement of the activity of 18:1-CoA thioesterase occurred when the solubilised fractions were dialyzed. Apparently, extensive solubilisation of 18:1-CoA thioesterase had occurred, but could not be detected because the high concentration of detergents present in the incubation mixture inhibited the thioesterase. At lower concentrations, however, the detergents had little effect on 18:1-CoA thioesterase (table 1). The 18:1-CoA:PC acyltransferase was also extensively solubilised by 0.5% (w/v) cholate, but this became apparent only after removal of the detergent by dialysis, although longer periods of dialysis (>18 h) resulted in a reduction of this activity (table 2).

18:1-PC desaturase activity was not detectable in either solubilised or non-solubilised fractions of pea leaf microsomes, irrespective of whether or not the fractions were dialyzed (table 2). Since the detergents, potentially inhibitory to the desaturase (table 1), had been removed by dialysis, the absence of 18:1-PC desaturase activity ought to be attributed to partial solubilisation of one or more components of the desaturase system; e.g., NADH:cytochrome b_5 reductase, cytochrome b_5 or the terminal 18:1-PC desaturase [9].

When the putative NADH:cytochrome b_5 reductase was assayed for as NADH:ferricyanide reductase [12] we found that more than 30% of this activity was solubilised (table 3). In contrast, although high activities of cytochrome b_5, assayed as cytochrome *c* reductase [4], were found in

Table 2

Metabolism of [1-^{14}C]oleoyl-CoA by dialyzed soluble and insoluble fractions obtained from pea leaf microsomes by detergent treatment

Detergent for solubilisation (conc. % w/v)	Microsomal fraction	Time of dialysis (h)	Radioactive 18:2 (% of total ^{14}C-labelled fatty acids)	Distribution of radioactivity in reaction (% of total)			
				FA	PC	Acyl-CoA	Others[a]
None	Whole microsomes	0	17.0	14.9	38.4	38.0	8.7
Cholate (0.5)	S	0	0.0	7.3	3.3	87.0	2.4
	NS	0	0.0	1.9	18.0	76.0	4.1
	S	18	0.0	68.0	10.1	16.0	5.9
	NS	18	0.0	4.4	4.6	90.0	1.0
	S	43	0.0	72.8	5.6	20.0	1.6
	NS	43	0.0	6.9	5.6	86.0	1.5
n-Octyl glucoside (1.5)	S	0	0.0	8.8	3.8	84.0	3.4
	NS	0	0.0	4.2	2.6	93.0	0.2
	S	18	0.0	70.2	5.5	22.0	2.3
	NS	18	0.0	4.8	4.8	90.0	0.4
	S	43	0.0	65.1	5.2	26.0	3.7
	NS	43	0.0	8.6	4.5	85.0	1.9

[a] Other minor products detected include diacylglycerols, triacylglycerols, lysophosphatidylcholines and phosphatidylethanolamines

Microsomes, isolated from leaves of 8-day old pea seedlings, were treated with detergent solutions for 2 h, and the solubilised (S) and non-solubilised (NS) fractions were separated by centrifugation at 100000 × *g* for 2 h. Both S and NS fractions were dialyzed for different periods, then incubated for 1 h with [1-^{14}C]18:1-CoA, and the products were analyzed as described in section 2

Table 3

Solubilisation of membrane-bound enzymes of oleate metabolism in pea leaf microsomes

Enzymatic activity	Activity expressed as nmol.mg protein^{-1}.h^{-1} (% of total)			Total recovery of original activity (%)
	Total microsomes	Solubilised fraction	Non-solubilised fraction	
Oleoyl-CoA thioesterase	7.1	6.4 (96)	0.3 (4)	95
Oleoyl-CoA:phosphatidylcholine acyltransferase	18.3	12.3 (73)	4.5 (27)	92
NADH:ferricyanide reductase	6.2	1.8 (30)	4.2 (70)	97
NADH:cytochrome *c* reductase	473	19 (5)	407 (95)	90

Microsomes were isolated from 8-day old pea seedlings, solubilised in the presence of 0.5% (w/v) cholate, and non-solubilised material was separated by centrifugation at 100000 × *g* for 2 h as described in section 2. Both solubilised and non-solubilised fractions were dialyzed for 18 h against 10 mM potassium phosphate buffer (pH 7.2) and assayed separately for 18:1-CoA thioesterase and 18:1-CoA:PC acyltransferase [3], cytochrome b_5 reductase [12] and cytochrome b_5 [5]

microsomes either in the presence or absence of detergent, less than 5% of this activity was solubilised under the conditions employed here (table 3). It was not possible to test for the solubilisation of the terminal 18:1-PC desaturase in the absence of a suitable reconstitution system containing the purified electron transfer components. However, it is possible that a part or all of the terminal desaturase was solubilised in our system. The absence of any 18:1-PC desaturase activity, as assayed for using 18:1-CoA as substrate, in either solubilised or non-solubilised fractions following treatment of microsomes with 0.5% (w/v) cholate or 1.5% (w/v) *n*-octyl glucoside, was probably due to selective solubilisation or partial solubilisation of one or more components of this multi-enzyme system.

As shown in table 3, the incubation of pea leaf microsomes with 0.5% (w/v) cholate, followed by dialysis to remove the detergent, results in the solubilisation of 96% of the 18:1-CoA thioesterase and 75% of the 18:1-CoA:PC acyltransferase activities. The electron transport components of the oleate desaturase were more difficult to solubilise. We have recently succeeded in purifying the solubilised long-chain acyl-CoA thioesterase activity from pea leaf microsomes (unpublished). We found 3 forms of long-chain acyl-CoA thioesterase, one of which has 18:1-CoA as its preferred substrate, as judged by the maximum value of the ratio V_{max}/K_m. Further work is being directed towards the purification and characterization of the remaining enzymes of microsomal oleate metabolism and their eventual reconstitution in vitro as a complete oleate desaturase system.

ACKNOWLEDGEMENTS

Part of this work was financed by a grant from the Deutsche Forschungsgemeinschaft. Additional finances were provided by the Bundesanstalt für Fettforschung, D-4400 Münster, and the Agricultural Research Council, UK.

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