Phospholipid and fatty acid composition in mitochondria from spinach (*Spinacia oleracea*) leaves and petioles

A comparative study

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Essentially chlorophyll-free mitochondria from photosynthetic (leaf) and non-photosynthetic tissue (petiole) were isolated from spinach (*Spinacia oleracea*). Leaf mitochondria were found to contain more phosphatidylcholine than phosphatidylethanolamine compared with petiole mitochondria. Galactolipids were found in small and equal amounts (5 mol of galactolipids/100 mol of galactolipids and phospholipids) in both leaf and petiole mitochondria. Fatty acid composition showed a significant difference in the amounts of $C_{18:2}$ and $C_{18:3}$ acids. The $C_{18:2}/C_{18:3}$ ratio was more than twice as high in all of the phospholipids studied from petiole mitochondria compared with the ratio in leaf mitochondria. More than 50% (mol/100 mol) of the fatty acids in the major lipids (phosphatidylcholine, phosphatidylethanolamine and cardiolipin) in petiole mitochondria were $C_{18:2}$. In the minor lipids (phosphatidylinositol and phosphatidylglycerol), $C_{16:0}$ dominated in both leaf and petiole mitochondria.

INTRODUCTION

Mitochondria have been purified from a number of plants [see Gardeström & Edwards (1985) for a brief review]. Although the lipid and fatty acid compositions for some of those purified mitochondria have been investigated in detail [see Harwood (1985) for a concise review], only one study has been reported for highly purified mitochondria from green tissue (Fuchs *et al.*, 1981). This lack of lipid studies on mitochondria from photosynthetic tissue is mainly due to difficulties in the removal of contaminating chloroplastic material from preparations of mitochondria. The thylakoid membrane fragments cover a broad range of sedimentation rate coefficients and isopycnic densities, including those for mitochondria. By use of aqueous two-phase systems, which separate according to surface properties, a great deal of contaminating thylakoid fragments can be removed (Gardeström *et al.*, 1978). Our laboratory has developed a preparative procedure which gives essentially chlorophyll-free mitochondria from green tissue in good yield, by combining differential centrifugation, phase partition and Percoll-density-gradient centrifugation (Bergman *et al.*, 1980).

This procedure has made it possible to make comparative studies of mitochondria isolated from different tissues of the same plant. It has thus been shown that mitochondria from spinach (*Spinacia oleracea*) leaves are different in their content of cytochromes, protein and lipids, compared with mitochondria from petioles (Gardeström *et al.*, 1983). Such differences in the composition of the mitochondrial membranes and matrices may reflect differences in the mitochondrial function. One obvious difference between mitochondria from photosynthetic and non-photosynthetic tissue is that the former has the ability to oxidize glycine and thus to participate in the photorespiration (Gardeström *et al.*, 1980).

The present paper reports the first study on comparative lipid and fatty acid composition of purified

mitochondria from different parts of the same plant. The lipid and fatty acid compositions were found to be different in mitochondria from photosynthetic tissue (spinach leaves) compared with mitochondria from non-photosynthetic tissue (spinach petioles). The consequences of those differences are discussed with respect to mitochondrial functions.

MATERIALS AND METHODS

Plant material

Spinach (*Spinacia oleracea* L., cv. Viking II) was grown in a nutrient solution (Siegenthaler & Depe'ry, 1976), with double the Fe concentration, at 18 °C under artificial light ($80 \mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$) with a 12 h light/12 h dark regime. Fully expanded leaves with mid-vein removed were used for the preparation of leaf mitochondria. The petioles were taken from plants with fully expanded leaves, of the same age as the plants used for the preparation of leaf mitochondria.

Preparation of mitochondria

Mitochondria from leaves and petioles were prepared by a three-step preparation procedure including differential centrifugation, phase partition and Percoll-density-gradient centrifugation (Bergman *et al.*, 1980). The composition of the phase system used was 6.2% (w/w) Dextran T500, 6.2% (w/w) poly(ethylene glycol) 3350, 0.3 M-sucrose, 2 mM-KCl and 5 mM-potassium phosphate buffer, pH 7.8. The thylakoid membranes remaining after the phase system partition were removed by discontinuous-Percoll-density-gradient centrifugation [21, 29 and 60% (w/v) Percoll; Pharmacia, Uppsala, Sweden]. Percoll was separated from the mitochondrial fraction by centrifugation twice at 20000 g for 10 min after dilution with at least 10 vol. of preparation medium. The final mitochondrial pellet was resuspended in distilled water to 1.3 ml; 100 μl was taken for protein determination (Lowry *et al.*, 1951; bovine

serum albumin as standard) and the rest was immediately extracted.

Enzyme assays

For the marker-enzyme assays the final mitochondrial pellet was resuspended in 0.5 ml of preparation medium. The intactness of the mitochondrial outer membrane was assayed by the succinate:cytochrome *c* oxidoreductase test, and cytochrome *c* oxidase (EC 1.9.3.1) was used as marker for mitochondria. Both activities were measured as described previously (Bergman *et al.*, 1980). Chloroplast-envelope contamination was investigated by the formation of galactolipids from UDP-[¹⁴C]galactose (Douce & Joyard, 1980). Peroxisomes were assayed by the presence of glycolate oxidase activity (EC 1.1.3.1) as described previously (Bergman *et al.*, 1980).

Extraction of lipids

Immediately after isolation, the mitochondrial suspension was extracted by a modified Bligh & Dyer procedure (Douce & Joyard, 1980); 4.5 ml of methanol/chloroform (2:1, v/v) was added to 1.2 ml of mitochondrial suspension and the mixture was centrifuged after 5 min. The residue was extracted with 1.5 ml of chloroform, and 1.5 ml of water was added to the combined extracts. The mixture was centrifuged and the chloroform layer withdrawn and evaporated to dryness in a stream of N₂. The lipid extract was dissolved in 1 ml of chloroform. Chlorophyll determination was done on the lipid extract as described by Comar & Zscheile (1942). The extract was stored at -20 °C under N₂ atmosphere.

Separation and quantification of lipids

The mitochondrial phospholipids were separated using two-dimensional t.l.c., mainly as described by Douce & Joyard (1980), and quantified by the method of Allen & Good (1971).

T.l.c. plates (silica gel G; Merck) were activated in 110 °C for 2 h and then cooled in an N₂ atmosphere. The lipids were applied quantitatively to the plates under a gentle jet of N₂. The plates were chromatographed, in the first direction in chloroform/methanol/water (65:25:4, by vol.) and in the second direction in chloroform/acetone/acetic acid/methanol/water (10:4:2:2:1, by vol.). After chromatography in the first direction, the plates were dried for 2 h under a slow stream of N₂. The lipid spots were revealed under u.v. light (360 nm) after staining with 0.2% (w/v) anilinonaphthalenesulphonate in methanol. Reference lipids and specific spray reagents were used for identification (Allen & Good, 1971). Phospholipids were purchased from Sigma, and glycolipids were kindly supplied by Dr. E. Selstam, Department of Plant Physiology, University of Umeå, Umeå, Sweden.

For quantification and determination of the fatty acid composition, spots from the t.l.c. plates were transferred to vials together with heptadecanoic acid as internal standard and then transesterified under an N₂ atmosphere (2 h, 68 °C) in H₂SO₄/benzene/methanol (1:1:20, by vol.). The fatty acid methyl esters were extracted with three successive 2 ml portions of n-hexane, and stored under nitrogen at -20 °C. The gas-chromatographic analyses were performed within 4 days after isolation of the fatty acids. A 2 m column (2 mm internal diameter)

packed with 10% diethyleneglycol succinate on Chromosorb Hp (100/120 mesh) was used for the analysis. Reference fatty acids (purchased from Sigma or obtained from spinach chloroplast membranes) were used for identification.

For neutral-lipid analysis, the lipid extract was separated by two-dimensional t.l.c. In the first direction chloroform/methanol/water (65:25:4, by vol.) was used to separate polar from neutral lipids, then hexane/diethyl ether/acetic acid (80:20:1, by vol.) was used in the second direction to separate the neutral lipids. Rhodamine 6G was used to reveal the spots (Schwertner & Biale, 1973).

RESULTS AND DISCUSSION

Preparation and purity of preparation

The yield of the mitochondrial preparations was, on average, 5.7 mg of mitochondrial protein from 200 g spinach leaves, and 3.0 mg of mitochondrial protein from 400 g of spinach petioles. The intactness of the outer membrane was $93 \pm 4\%$ (six experiments) for leaf mitochondria and $80 \pm 5\%$ (three experiments) for petiole mitochondria. The mitochondrial preparations were very pure. The most serious contamination was peroxisomes in the preparations from leaves, where the enrichment of mitochondrial relative to peroxisomes was more than 10-fold (Bergman *et al.*, 1980). Owing to the low lipid-to-protein ratio in peroxisomes (Donaldson *et al.*, 1972), the contamination seems to be insignificant. In the preparations from petioles the enrichment of mitochondria relative to peroxisomes was found to be 40 ± 10 times (two experiments) and the apparent specific glycolate oxidase activity 4 ± 2 nmol of O₂/min per mg of protein. The thylakoid contamination, expressed as the chlorophyll content, was very low in the preparations from leaves (0.2 ± 0.14 µg/mg of mitochondrial protein), but somewhat higher in the preparations from petioles (1.0 ± 0.5 µg/mg of mitochondrial protein). This is probably due to insufficient separation of mitochondria and chloroplast material in the two-phase separating step. It is known that differences in the surface properties of the membranes are of major importance in the distribution in the two-phase system. Such differences between mitochondria from leaves and petioles of spinach have been reported (Gardeström *et al.*, 1983). It is also known that mitochondria from leaf, petiole and root have different isoelectric points, measured as the cross-partition points in the two-phase systems (A. Bergman, personal communication; Albertsson *et al.*, 1982).

Galactolipid composition

The amounts of galactolipids found in the mitochondrial preparations from both leaves and petioles (3 ± 1.9 and 8 ± 5 µg/mg of mitochondrial protein respectively) were higher than those expected from the known galactolipid/chlorophyll ratio [1–2 according to Allen *et al.* (1972)] in thylakoid membranes. The galactolipids were identified as monogalactosyldiacylglycerol, digalactosyldiacylglycerol and sulphoquinovosyldiacylglycerol. The amounts of the different galactolipids were, on a molar-percentage basis of total amounts of galactolipids and phospholipids, equal in leaf and petiole mitochondria: $1.7 \pm 0.99\%$ for monogalactosyldiacyl-

glycerol, $2.8 \pm 0.83\%$ for digalactosyldiacylglycerol and $< 1.0\%$ for sulphoquinovosyldiacylglycerol. The fatty acid compositions of the galactolipids were similar in mitochondria from leaves and petioles and show the same pattern as reported for spinach chloroplast envelope and thylakoid membranes (Douce & Joyard, 1980). This could indicate that the presence of galactolipids might be due to plastid contamination (McCarty *et al.*, 1973; Ohmori & Mitsuhiro, 1974; Fuchs *et al.*, 1981).

The chloroplast-envelope contamination, assayed by the incorporation of UDP-galactose into galactolipids, was found to be low and identical in the leaf and petiole mitochondrial preparations. The enrichment of mitochondria relative to chloroplast envelope was, in the preparations from leaf, 80 ± 30 -fold (three experiments), and, in the preparations from petiole, 40 ± 20 -fold (four experiments). The apparent specific activities of galactolipid formation from UDP-galactose were, in the mitochondrial preparations from leaf, 1.1 ± 0.2 pmol/min per mg of protein (five experiments), and, from petiole, 0.9 ± 0.2 pmol/min per mg of protein (five experiments).

No traces of lysolipids or phosphatidic acid were detected on the t.l.c. plate. An unidentified lipid was found that contained fatty acids liberated by the transmethylation procedure. The amount was 1% of the total phospholipids (assuming two fatty acids in the molecule). The retention on the t.l.c. plates in the '65:25:4' system was approximately equal to that of digalactosyldiacylglycerol and in the '10:4:2:2:1' system slightly higher than that of monogalactosyldiacylglycerol.

An extract from leaf mitochondria (9.4 mg of protein) did not give any detectable amounts of neutral lipids (non-esterified fatty acids, mono-, di- and tri-acylglycerols, sterols and steryl esters) on a t.l.c. plate developed as described in the Materials and methods sections.

Phospholipid composition

The phospholipid composition of mitochondria from spinach resemble those found for other plant mitochondria from both photosynthetic (Fuchs *et al.*, 1981) and non-photosynthetic (McCarty *et al.*, 1973; Moreau *et al.*, 1974; Bligny & Douce, 1980) tissue. The differences obtained may be due to different methods used in preparation and analysis. The phospholipid composition in mitochondria from leaves and petioles was similar (Table 1), with one significant difference. Phosphatidylcholine was the predominant phospholipid in leaf mitochondria, but there were equal amounts of phosphatidylcholine and phosphatidylethanolamine in petiole mitochondria.

Fatty acid composition

The fatty acids found in both leaf and petiole mitochondria were, on a qualitative basis, the same as those found in a number of other mitochondria from green and non-green plant tissue (McCarty *et al.*, 1973; Moreau *et al.*, 1974; Bligny & Douce, 1980; Fuchs *et al.*, 1981). In mitochondria from oat (*Avena sativa*) mesophyll cells, phosphatidylglycerol was reported to contain the *cis* isomers, and no 3-*trans* isomer, of $C_{18:1}$ (Fuchs *et al.*, 1981). In spinach petiole mitochondria phosphatidylglycerol was found to contain no 3-*trans*-hexadecanoic acid but, as judged by retention time, 9-*cis*-hexadecanoic

Table 1. Phospholipid composition of spinach leaf and petiole mitochondria

For mitochondrial preparation, lipid extraction and characterization, see the Materials and methods section. Results are means $\pm$ s.d. for six and three independent determinations for leaf and petiole tissue respectively.

Phospholipid	Composition (mol/100 mol)	
	Leaf	Petiole
Phosphatidylcholine	45 ± 1.9	42 ± 0.8
Phosphatidylethanolamine	39 ± 3.6	42 ± 2.6
Diphosphatidylglycerol	11 ± 2.3	12 ± 1.8
Phosphatidylinositol	1 ± 0.5	2 ± 0.6
Phosphatidylglycerol	4 ± 1.5	2 ± 1.2

acid could be identified. In contrast, phosphatidylglycerol in leaf mitochondria most probably contained 3-*trans*-hexadecanoic acid, whereas only traces of 9-*cis*-hexadecanoic acid could be detected.

The fatty acid composition (Table 2) showed a high content of polyunsaturated fatty acids (70–80%), in agreement with other reports on the fatty acid composition of mitochondria from photosynthetic and non-photosynthetic tissue. One interesting observation is, however, that the amounts of $C_{18:2}$ and $C_{18:3}$ were significantly different in leaf and petiole mitochondria. In the major lipids (phosphatidylcholine, phosphatidylethanolamine and cardiolipin), $C_{18:2}$ is predominant in petiole mitochondria, but in leaf mitochondria $C_{18:3}$ is predominant. Both in leaf and petiole mitochondria, $C_{16:0}$ is the predominant fatty acid in the minor lipids (phosphatidylinositol and phosphatidylglycerol), but the same difference in the ratio of $C_{18:2}$ to $C_{18:3}$ is, however, also found in these lipids when leaf and petiole are compared.

The fatty acid composition in plants is reported to vary considerably according to environmental factors such as light, temperature (Harwood, 1980; Hartmann *et al.*, 1981; Harwood, 1985) and age (Matsuzaki *et al.*, 1983). The plant material used in these studies was of the same age and grown under controlled identical conditions with respect to light, temperature, humidity and nutrient solution. It is thus unlikely that the differences obtained in this work are due to environmental factors.

Total fatty acid analysis of whole plant cells has shown that the major fatty acid is $C_{18:3}$ in both green and non-green leaves of barley (*Hordeum vulgare*) mutants (Dorne *et al.*, 1982). In non-leaf tissue of tobacco (*Nicotiana tabacum*) it has been shown that the major fatty acid is $C_{18:2}$ (Matsuzaki *et al.*, 1983). This indicates that the fatty acid composition may be dependent on plant tissue. Our results reported here show that both the phospholipid and fatty acid compositions in mitochondria from spinach leaves and petioles are rather similar to those reported for other plant mitochondria. Some interesting differences may, however, be mentioned. The leaf mitochondria contain more phosphatidylcholine than phosphatidylethanolamine, and more $C_{18:3}$ than $C_{18:2}$ compared with petiole mitochondria.

It is known that the organization of membranes is dependent on the structure and conformation of the membrane lipids, e.g. size of polar head, length and

Table 2. Fatty acid composition of individual phospholipids in mitochondria from spinach leaf and petiole tissue

For mitochondrial preparations, lipid extraction and characterization, see the Materials and methods section. Fatty acids are characterized by numbers of carbon atoms (x) and double bonds (y), e.g. $C_{x:y}$. $C_{16:1}$ fatty acid in phosphatidylglycerol was identified as 3-*trans*-hexadecanoic acid and as 9-*cis*-hexadecanoic acid in petiole mitochondria. Results are means $\pm$ s.d. for six and three independent preparations of mitochondria from leaf and petiole tissue respectively. Abbreviation: tr, trace.

Lipid	Tissue	Fatty acid...	Composition (mol/100 mol)					
			$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
Phosphatidylcholine	Leaf		9.5 $\pm$ 0.8	tr	tr	9.0 $\pm$ 0.7	35 $\pm$ 2.0	47 $\pm$ 2.6
	Petiole		9.2 $\pm$ 0.8	1.2 $\pm$ 0.3	tr	5.6 $\pm$ 0.8	50 $\pm$ 1.8	34 $\pm$ 1.9
Phosphatidylethanolamine	Leaf		9 $\pm$ 2.1	tr	tr	6 $\pm$ 1.2	48 $\pm$ 1.9	37 $\pm$ 2.8
	Petiole		9 $\pm$ 3.3	1.0 $\pm$ 0.5	tr	5 $\pm$ 2.0	59 $\pm$ 6.5	25 $\pm$ 2.0
Cardiolipin	Leaf		1.6 $\pm$ 0.3	tr	tr	7 $\pm$ 1.1	38 $\pm$ 1.6	53 $\pm$ 2.1
	Petiole		1.4 $\pm$ 0.2	tr	tr	5.0 $\pm$ 0.2	56 $\pm$ 9.2	38 $\pm$ 7.0
Phosphatidylinositol	Leaf		50 $\pm$ 13	tr	tr	6.0 $\pm$ 0.8	18 $\pm$ 2.5	26 $\pm$ 5.0
	Petiole		32 $\pm$ 1.4	3.0 $\pm$ 0.5	tr	7 $\pm$ 2.2	36 $\pm$ 1.4	22 $\pm$ 0.7
Phosphatidylglycerol	Leaf		36 $\pm$ 5.6	12 $\pm$ 3.2	2.1 $\pm$ 0.8	4.4 $\pm$ 0.9	14 $\pm$ 2.9	32 $\pm$ 1.3
	Petiole		43 $\pm$ 3.02	6 $\pm$ 1.2	tr	5 $\pm$ 3.6	29 $\pm$ 3.0	18 $\pm$ 2.6

unsaturation of the fatty acids (Seelig & Seelig, 1980). The organization will also be modulated by membrane proteins. Different types of proteins (with respect to size, shape and charge) give different effects on the lipid packing, e.g. possibility to form stable membranes (Israelachvili *et al.*, 1980; Taraschi *et al.*, 1982; Van Echteld *et al.*, 1980). It thus seems possible that quantitative and qualitative changes in membrane proteins will cause changes in the content of specific lipids in order to stabilize the membrane.

A number of functional and structural differences between leaf and petiole mitochondria are known: (a) only leaf mitochondria can oxidize glycine in the photorespiratory pathway (Gardeström *et al.*, 1980); (b) leaf mitochondria have a higher membrane-protein/lipid ratio and a lower cytochrome *c*/protein ratio than have petiole mitochondria (Gardeström *et al.*, 1983); (c) a lower content of respiratory chain is present in leaf mitochondria (Gardeström *et al.*, 1983).

It is tempting to speculate that differences in membrane proteins in relation to at least some of the functional and structural differences mentioned may cause the observed different lipid compositions in leaf and petiole mitochondrial.

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