Effects of glucose starvation on the oxidation of fatty acids by maize root tip mitochondria and peroxisomes: evidence for mitochondrial fatty acid β-oxidation and acyl-CoA dehydrogenase activity in a higher plant

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Fatty acid β-oxidation was studied in organellar fractions from maize root tips by h.p.l.c. and radiometric analysis of the products of incubations with [1-^{14}C]octanoate and [1-^{14}C]palmitate. In crude organellar fractions containing both mitochondria and peroxisomes, octanoate and palmitate β-oxidation, as determined by the production of acetyl-CoA, was functional and, for palmitate, was activated 4–12-fold after subjecting the root tips to 48 h of glucose starvation. The sensitivity to a 'cocktail' of respiratory-chain inhibitors containing cyanide, azide and salicylhydroxamate depended on the conditions of incubation, with no inhibition in a medium facilitating peroxisomal β-oxidation and a significant inhibition in a medium potentially facilitating mitochondrial β-oxidation. Indeed, preparations of highly purified mitochondria from glucose-starved root tips were able to oxidize octanoate and palmitate to give organic acids of the tricarboxylic acid cycle. This activity was inhibited 5–10-fold by the above cocktail of respiratory-chain inhibitors, with no parallel accumulation of acetyl-CoA, thus showing that the inhibition affected β-oxidation rather than the pathway from acetyl-CoA to the organic acids. This provides the first evidence that the complete β-oxidation pathway from fatty acids to citrate was functional in mitochondria from a higher plant. Moreover, an acyl-CoA dehydrogenase activity was shown to be present in the purified mitochondria. In contrast with the peroxisomal activity, mitochondrial β-oxidation showed the same efficiency with octanoate and palmitate and was strictly dependent on glucose starvation.

INTRODUCTION

Carbohydrates are generally thought to be the major respiratory substrates in plant cells (ap Rees, 1990). However, in certain plant materials, such as germinating lettuce or sunflower seeds, it has been shown, either through steady-state labelling *in vivo* and modelling (Salon et al., 1988) or through metabolic studies *in organello* (Raymond et al., 1992), that the β-oxidation of fatty acids was functional and could provide acetyl-CoA directly for respiration. Furthermore, when carbohydrate starvation is carried out under controlled conditions, as can be achieved with excised maize root tips (Saglio and Pradet, 1980), it has been shown that the activity of fatty acid β-oxidation was increased at least 2.5-fold under the effects of the glucose-starvation treatment (Dieuaide et al., 1992). In contrast with animal tissues (see Rognstad, 1991), some degree of uncertainty surrounds the subcellular localization of fatty acid β-oxidation in higher plants (see Miernyk et al., 1991). It was considered as exclusively peroxisomal (Harwood, 1988; see also Schulz, 1991), although palmitoylcarnitine had been reported to be a respiratory substrate for avocado mitochondria (Panter and Mudd, 1973). More recent studies confirmed that the respiration of mitochondria from pea cotyledons (Wood et al., 1984) and from avocado pears (Masterson et al., 1992) was stimulated by the addition of palmitate or palmitoyl-CoA only in the presence of exogenous carnitine. These respiration studies may be difficult to interpret because of the activating effects of CoASH on the respiration of malate (Day et al., 1984), which was used as starting respiratory substrate, and because of the uncoupling effects of fatty acids on plant mitochondria (Macri et al., 1991). However, mitochondria from some plant tissues such as pea cotyledons (Wood et al., 1986) were shown to contain at least some of the enzymes of β-oxidation. Furthermore, in the case of enoyl-CoA hydratase, an isoenzyme immunologically distinct from the peroxisomal enzyme was partially purified from pea cotyledon mitochondria (Miernyk et al., 1991). Thus it remained to be ascertained that higher-plant mitochondria could carry out the complete β-oxidation pathway, i.e. the conversion of fatty acids into acetyl-CoA.

Excised maize root tips show an integrated metabolic response to glucose starvation (Brouquisse et al., 1991, 1992; Dieuaide et al., 1992), with a potential contribution from mitochondria (Couée et al., 1992). Thus the role of mitochondria could have been the more efficient utilization of citrate (Raymond et al., 1992), malate (Dieuaide et al., 1992) and succinate (see Huang et al., 1983) that may be produced by peroxisomal β-oxidation. However, the ability of 'glucose-starved mitochondria' to oxidize exogenously supplied substrates was shown to be similar to that of 'non-starved mitochondria' (Couée et al., 1992). This prompted us to envisage the possibility of β-oxidation occurring in glucose-starved mitochondria. The first step of β-oxidation consists of the formation of a C-2–C-3 double bond which, as described in animal mitochondria, is catalysed by acyl-CoA dehydrogenases transferring electrons to an electron-transferring flavoprotein which feeds reducing equivalents to the respiratory chain (see Engel, 1992). The first step of β-oxidation in peroxisomes is catalysed by acyl-CoA oxidases, whose flavin moiety is re-oxidized directly by oxygen (see Osmundsen et al., 1991). Thus the use of inhibitors of the respiratory chain, which, in plant mitochondria, consists of the cyanide-sensitive electron pathway and the cyanide-resistant, salicylhydroxamate (SHAM)-sensitive, electron pathway (see Douce, 1985), allows one to

Abbreviations used: DCPIP, 2,6-dichlorophenol-indophenol; DTNB, 5,5′-dithiobis-(2-nitrobenzoate); NEM, *N*-ethylmaleimide; PMS, phenazine methosulphate; SHAM, salicylhydroxamate; TNB^{2-}, 2-nitro-5-thiobenzoate.

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distinguish between mitochondrial and peroxisomal β-oxidation activities. Furthermore, in the mitochondria, the import of fatty acids is dependent on the presence of carnitine (see Schulz, 1991), thus giving further possibilities to distinguish between mitochondrial and peroxisomal β-oxidation. We have studied higher-plant β-oxidation by h.p.l.c. analysis and subsequent radiometric measurement of the acyl-CoA esters and organic acids obtained after incubation of crude peroxisomal preparations or purified mitochondria with radiolabelled fatty acids. Through the use of inhibitors and of characteristic incubation conditions, we show that, in maize root tips, both peroxisomal and mitochondrial β-oxidation activities are functional and are differently activated by carbohydrate starvation. We also show the presence of acyl-CoA dehydrogenase activity.

MATERIALS AND METHODS

Plant material and glucose-starvation treatment

Maize seeds (*Zea mays* L., cv. DEA, Pioneer France Maïs, France) were germinated at 25 °C in the dark for 3 days between sheets of Whatman 3MM chromatography paper (Whatman International, Maidstone, Kent, U.K.) soaked in the mineral nutrient medium described by Saglio and Pradet (1980). The 3 mm-long tips of seminal roots were excised and either used immediately for the preparation of organelles or incubated for glucose-starvation treatment. For the latter, the excised root tips were incubated at 25 °C in the mineral nutrient medium supplemented with 1 % (v/v) of the antibiotic and antimycotic mixture A 7292 from Sigma (St. Louis, MO, U.S.A.) and 0.1 M Mes/KOH (pH 6.0). O_2/N_2 (1:1) was continuously bubbled through the incubation medium to maintain a partial oxygen pressure above 35 kPa, which is the critical oxygen pressure for maize roots in aqueous solutions (Saglio et al., 1984).

Chemicals

Analytical-grade salts were purchased from Merck (Darmstadt, Germany). Solvents of h.p.l.c. grade were from Prolabo (Paris, France). Tetrabutylammonium hydroxide was obtained from Sigma and converted into tetrabutylammonium phosphate as described by Baker and Schooley (1979). Percoll was from Pharmacia (Uppsala, Sweden). The γ-globulin standard was purchased from Calbiochem (San Diego, CA, U.S.A.). The substrates [1-^{14}C]palmitic acid (1.99 GBq/mmol), [1-^{14}C]octanoic acid (2.15 GBq/mmol) and [^{3}H]acetyl-CoA (122.1 GBq/mmol) were purchased from NEN (Paris, France) or Dositech (Paris, France). All other reagents and enzymes, unless otherwise specified, were obtained from Sigma.

Preparation of organellar fractions

Peroxisome-containing fractions and mitochondria were prepared from freshly excised root tips or from root tips which had been subjected to 48 h of glucose-starvation treatment. The organellar fraction containing peroxisomes was obtained by centrifugation of a crude cellular extract on a one-step (35 and 60 %, w/w) sucrose gradient as described by Dieuaide et al. (1992). This organellar fraction was in a volume of 6 ml at a final protein concentration of approx. 2 mg/ml. The assay of peroxisomal and mitochondrial enzyme markers at different stages of the isolation procedure indicated that both peroxisomes and mitochondria were enriched in this preparation, with a 2.5-fold enrichment factor.

The isolation of low- and high-density mitochondria was carried out by differential and Percoll-gradient isopycnic centrifugation as previously described (Couée et al., 1992), except that Percoll-purified mitochondria were diluted and, after centrifugation, resuspended in washing medium without BSA. In the present work, only the mitochondria of high density, which are more differentiated, with many cristae and a dense matrix, were used for the assay of β-oxidation. Non-starved and glucose-starved mitochondrial preparations were resuspended in 0.1 ml of washing medium at final protein concentrations of 6 ± 2 and 14 ± 3 mg/ml respectively (mean $\pm$ S.E.M., for at least five separate experiments). The latency of matrix enzyme markers was 95 %, thus showing the integrity of the purified mitochondria. Hypo-osmotically damaged mitochondria were obtained by pelleting 40 μl portions of mitochondrial preparation at 12000 $g_{max.}$ for 15 min and resuspending the pellets in 160 μl of 10 mM potassium phosphate buffer, pH 7.2, containing 1 mM EDTA. There was no loss of protein in the course of this additional step, in accordance with the high integrity of the preparations.

Enzyme activities

All enzyme activities were assayed spectrophotometrically at 30 °C, unless otherwise specified, by previously published methods. All assays were first performed on blanks containing all the constituents of the assay except the substrate which was added to initiate the reaction. Activities were linear with respect to time for at least 2 min and were proportional to the amounts of sample protein added to the assay. The activities of fumarase (EC 4.2.1.2), catalase (EC 1.11.1.6) and urate oxidase (EC 1.7.3.3) were assayed as described by Hill and Bradshaw (1969), Aebi (1987) and Hong and Schopfer (1981) respectively. Acyl-CoA oxidase (EC 1.3.3.6) and 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) were assayed as described by Gerhardt (1987). Acyl-CoA dehydrogenase (EC 1.3.99.2,3) activity was assayed in terms of the reduction of 2,6-dichloroindophenol (DCPIP) as an electron acceptor and phenazine methosulphate (PMS) as an intermediate electron carrier (see Izai et al., 1992). The decrease in A_{600} was monitored in a reaction mixture containing 50 mM Hepes/KOH, pH 7.5, 1 mM KCN, 1 mM SHAM, 120 μg/ml DCPIP, 120 μg/ml PMS, the enzyme sample and 50 μM butyryl-CoA, octanoyl-CoA or palmitoyl-CoA in a final volume of 1.1 ml. The reaction was started by addition of the acyl-CoA substrate. Blanks in the absence of enzyme sample were carried out to assess the rate of reduction by contaminating CoASH. This activity was assayed at 25 °C in order to minimize the background rate. The activity of acyl-CoA esterase (EC 3.1.2.2) was assayed in the presence of 0.15 mM 5,5′-dithiobis-(2-nitrobenzoate) (DTNB) under the same conditions, except that DCPIP and PMS were omitted, by measuring the appearance of 2-nitro-5-thiobenzoate (TNB^{2-}) at 412 nm. The absorption coefficient of TNB^{2-} was taken to be 14150 $M^{-1} \cdot cm^{-1}$ (Riddles et al., 1983). This activity was also assayed at 25 °C, as it served as a control of the PMS/DCPIP assay. One enzyme unit was defined as that catalysing the formation of 1 μmol of product in 1 min under the stated conditions.

Assay of protein

Protein contents were determined by the method of Bradford (1976) with bovine γ-globulin as the standard. Whenever necessary, the content of BSA in the extraction and washing mediums, expressed as the equivalent in bovine γ-globulin, was subtracted from the protein content. The added BSA represented 10–50 % of the total protein content.

Assay of carnitine

The carnitine content in mitochondrial preparations was determined by the radiometric method described by McGarry and Foster (1985). The carnitine acetyltransferase preparation used in this assay was obtained from Boehringer Mannheim (Germany).

Radiochemical assays of β-oxidation

Incubations with 1-^{14}C-labelled fatty acid substrates

β-Oxidation assays were carried out in reaction mixtures designed to facilitate mitochondrial (reaction medium M) or peroxisomal (reaction mixture P) β-oxidation. Both media contained 175 mM Tris/HCl, pH 8.5, 15 mM $MgCl_2$, 1 mM ATP, 1 mM NAD^+, 10 mM phenylmethanesulphonyl fluoride, 25 μM leupeptin and 17 μM chymostatin. These protease inhibitors were necessary to ensure the linearity of the assay (Dieuaide et al., 1992). Reaction medium M also contained 300 mM mannitol, 0.1 mM ADP, 20 mM malate, 2 mM dithiothreitol, 0.1 mM carnitine, 30 μM CoASH, unless otherwise specified, and BSA at a molar ratio of 1.67 relative to the concentration of fatty acid substrate (Mannaerts et al., 1979). Reaction mixture P also contained 1 mM CoASH. Whenever required, potential effectors of β-oxidation were added to the reaction medium as specified in the Tables. The assay was carried out with respectively 100 μl, 10 μl or 40 μl of the peroxisome-containing fraction, the mitochondrial preparation or the hypo-osmotically damaged mitochondrial preparation. When medium M was used, mitochondria were preincubated for 10 min at 25 °C in this medium in the absence or in the presence of the required effectors. In all cases, the reaction was initiated by addition of [1-^{14}C]palmitic acid or [1-^{14}C]octanoic acid at 73 kBq (±3, range) per assay and at a concentration of 35 μM (±2, range) as fatty acid substrates. Incubations were carried out at 25 °C in a final volume of 1 ml in 20 ml glass vials. The reaction was stopped by acidification and refrigeration with 0.15 ml of ice-cold 1 M HCl and 0.6 ml of ice-cold 0.5 M potassium phosphate buffer, pH 3, and the samples were kept at 0–4 °C. Internal standards of palmitoyl-CoA or octanoyl-CoA (50 nmol per assay) and [^{3}H]acetyl-CoA (1 kBq per assay) were added in order to determine the yields during the purification of these acyl-CoA esters. The samples were then frozen and stored at −20 °C. Zero-time assays were performed by adding the organellar samples after acidification and refrigeration of the reaction mixture.

Purification of acyl-CoA esters and organic acids

Acyl-CoA esters and organic acids were purified from the reaction mixture by hydrophobic-interaction chromatography and ion-exchange chromatography. Each sample was applied to a Sep-Pak C_{18} cartridge (Millipore, Milford, MA, U.S.A.) as described by Raymond et al. (1992). The effluent plus the washes with 20 ml of 1 mM HCl from the Sep-Pak cartridge were collected, thus giving the fraction of polar components containing the organic acids. This polar fraction was evaporated to dryness under vacuum and then dissolved in 500 μl of water. Organic acids were further purified from the polar fraction by passing through a 3 ml column of Dowex 1 X8-200 (formate form) (Sigma). The column was rinsed with 15 ml of water and the organic acids were eluted with 12 ml of 4 M formic acid. The elutate was evaporated to dryness. The residue was taken up in 0.5 ml of water and the pH was adjusted to 4 with 2 M NaOH. Methanol (1.5 ml) and chloroform (3 ml) were then added in order to eliminate residual phospholipids. After vortex-mixing and centrifugation, the upper phase was recovered. Water (0.5 ml) was added to the apolar phase, and, after mixing and centrifugation, a second upper phase was recovered and added to the first. This polar phase, containing the organic acids, was evaporated to dryness under vacuum and stored at −20 °C.

The acyl-CoA esters were eluted from the Sep-Pak C_{18} cartridge with 4 ml of 10 mM tetrabutylammonium phosphate in methanol/water (9:1, v/v) as described by Raymond et al. (1992). Each sample of acyl-CoA eluate was collected into a glass tube, concentrated to about 0.1 ml under nitrogen and then stored at −20 °C.

Analysis by h.p.l.c. and radiometric assay of acyl-CoA esters and organic acids

Both the acyl-CoA fraction and the organic acid fraction were analysed by h.p.l.c. using a Vista 5500 liquid-chromatography system (Varian, Palo Alto, CA, U.S.A.) with a Spherisorb (Phase Separations Ltd., U.K.) C_8 (5 μM) column (0.4 cm inner diameter × 20 cm length) as previously described (Raymond et al., 1992). Portions (40 μl) of the acyl-CoA fraction were applied to the column and then eluted with an acetonitrile gradient; 1 ml fractions were collected. The dry organic acid fractions were taken up in 50 or 100 μl of water, and 40 μl portions were immediately applied to the column. Elution was performed with 10 mM potassium phosphate buffer, pH 2.5; 1 ml fractions were collected. The radioactivity of each eluted fraction was measured in a Packard scintillation counter with the conventional ^{3}H/^{14}C dual-label protocol. The radioactivity obtained with the extracts from zero-time incubations or from incubations with mitochondria which had been subjected to 100 °C for 10 min varied between 50 and 200 d.p.m. per fraction. These background radioactivities were subtracted from the values of assay incubations.

Assay of CoASH and CoAS-SCoA

The procedure used to purify and analyse CoASH and CoAS-SCoA was similar to the method of separation of acyl-CoA esters, but with some modifications to optimize the separation. The Sep-Pak cartridge was eluted with 2 ml of ethanol/water (3:2, v/v) containing 10 mM ammonium phosphate as described by Hosokawa et al. (1986). The gradient for h.p.l.c. was also modified. The column was eluted during 5 min with a mixture of 50 mM potassium phosphate, pH 5.3, and acetonitrile (19:1, v/v). Acetonitrile was then increased linearly to 75% in 15 min, and the column was re-equilibrated with the initial solvent for 10 min before the next injection. The elution was found to be extremely sensitive to pH variations in the samples, and therefore the identification of elution peaks was confirmed by internal standards of CoA. The elution profiles were monitored spectrophotometrically at 254 nm with an on-line detector.

RESULTS

Characterization of the β-oxidation activity of a peroxisome-containing fraction from maize root tips

The peroxisome-containing fraction was isolated as described in the Materials and methods section from approx. 300 maize root tips either before or after 48 h of glucose-starvation treatment. The yields of protein, fumarase activity and 3-hydroxyacyl-CoA dehydrogenase activity were similar before and after the glucose-starvation treatment, thus allowing the comparison of the activities per ml of organellar preparation (see Dieuaide et al., 1992). This fraction, when incubated in medium P, was able to oxidize octanoate or palmitate (Figure 1) to acetyl-CoA. No

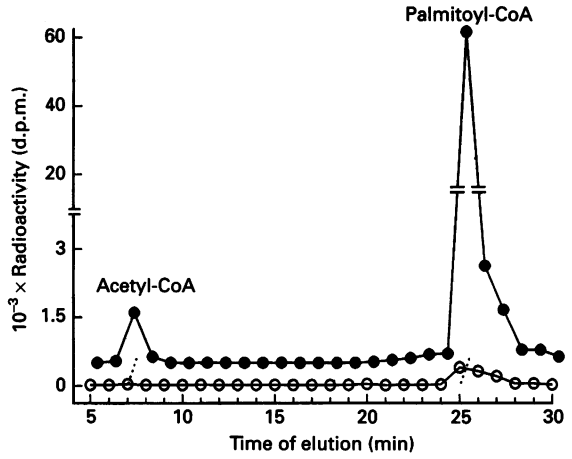

Figure 1 Analysis of radiolabelled acyl-CoA esters after incubation of a peroxisome-containing fraction from glucose-starved maize root tips with [1-^{14}C]palmitate

A sample of the peroxisome-containing fraction from 300 glucose-starved maize root tips was incubated with [1-^{14}C]palmitate for 0 min (○) or 30 min (●) in medium P as described in the Materials and methods section. The acyl-CoA esters were then purified and analysed by h.p.l.c. and subsequent radiometric measurement. A typical profile of elution is given. Octanoyl-CoA was eluted at 18.8 min (results not shown).

significant production of radiolabelled organic acids was observed, thus showing that the citrate synthase- and malate synthase-catalysed reactions (see Dieuaide et al, 1992) did not take place. Table 1 shows the production of radiolabelled substrate octanoyl-CoA and palmitoyl-CoA and of acetyl-CoA by this peroxisome-containing fraction in the absence or presence of a cocktail or inhibitors of the respiratory chain containing cyanide, azide and SHAM. This cocktail was added at high concentrations and at regular intervals in order to ensure a complete inhibition of respiration and also to disclose any non-specific effect on peroxisomal β-oxidation. This cocktail had generally no appreciable inhibitory effect on the levels of substrate fatty acyl-CoA esters (Table 1), and an activating effect on the β-oxidation activity. In the case of octanoate β-oxidation by non-starved preparations, the 30% inhibition of acetyl-CoA production could be related to the 30% inhibition of octanoyl-CoA production.

These results did not preclude the existence of mitochondrial β-oxidation, because, in medium P, any mitochondrial β-oxidation may have been hampered by the disruption of mitochondria under hypo-osmotic conditions (see Mannaerts et al., 1979) and by the absence of respiratory substrates and ADP (see Wood et al., 1984; Sabbagh et al., 1985). Thus, in a preliminary experiment, the β-oxidation activity of a peroxisome-containing fraction was measured in medium M, which is characterized by iso-osmotic conditions and the presence of carnitine, ADP and malate, and thus suitable for mitochondria. [1-^{14}C]Palmitate was used as substrate, and the production of radiolabelled palmitoyl-CoA, acetyl-CoA and organic acids was monitored in the absence and in the presence of respiratory-chain inhibitors as described above. The level of substrate palmitoyl-CoA was 310000 d.p.m., and β-oxidation gave 3800 d.p.m. of acetyl-CoA and 7400 d.p.m. of organic acids after 30 min of incubation. In this case, i.e. in the presence of added malate, a number of differences were observed: there was a significant production of radiolabelled organic acids; the β-oxidation was lower than under the conditions of medium P, which might reflect the lower level of CoASH or the limitation of palmitoyl-CoA import into intact organelles; the production of acetyl-CoA and organic acids from palmitate was significantly affected by the cocktail of respiratory-chain inhibitors. The production of substrate palmitoyl-CoA decreased by 25%, whereas there was a 25% inhibition of acetyl-CoA production and a 65% inhibition of organic acid production. Thus it appeared that, under the assay conditions of medium M, a fraction of the production of β-oxidation end-products was dependent on the respiratory chain.

β-oxidation activity in purified mitochondria from glucose-starved maize root tips

High-density mitochondria, of apparent density 1.070 g/ml, were isolated from approx. 1000 glucose-starved maize root tips as described in the Materials and methods section. Electron microscopy (Couée et al., 1992) has shown that the protocol used in the present study yielded mitochondrial preparations containing no intact peroxisomes and no intact bacteria. The enrichment in mitochondrial enzyme markers was between 4- and 6-fold, in agreement with the results of Couée et al. (1992),

Table 1 Production of acyl-CoA esters after incubation of a peroxisome-containing fraction from maize root tips with [1-^{14}C]octanoate or [1-^{14}C]palmitate

Peroxisome-containing fractions from 300 maize root tips before (non-starved) and after (starved) 48 h of glucose-starvation treatment were prepared as described in the Materials and methods section. Approx. 290 μg of protein of the peroxisome-containing fraction was incubated with [1-^{14}C]octanoate or [1-^{14}C]palmitate for 30 min in medium P (1 ml) in the absence or in the presence of respiratory-chain inhibitors. The cocktail of respiratory-chain inhibitors consisted of 2 mM KCN, 1 mM NaN_3 plus 2 mM SHAM. This cocktail was added three times at 10 min intervals. Addition of the equivalent amount of dimethyl sulphoxide, in which SHAM was dissolved, had no effect. The radiolabelled products of these incubations were analysed by h.p.l.c. and radiometric measurement. The production of acetyl-CoA was linear with time for at least 40 min. The results of control incubations are the values from two independent experiments. The results of the incubations with respiratory-chain inhibitors are means (±range; $n = 2$) of the percentages of the corresponding control experiments, except in one case where only one value is given.

		Production of acyl-CoA esters			
		Control (10^{-3} × d.p.m./ml of peroxisome-containing fraction)		In the presence of respiratory-chain inhibitors (% of control)	
Treatment	Fatty acid	Fatty acid CoA ester	Acetyl-CoA	Fatty acid CoA ester	Acetyl-CoA
Non-starved	[1-^{14}C]octanoate	110–330	20–28	72±8	69±5
	[1-^{14}C]palmitate	7200–8700	70–18	76±14	180
Starved	[1-^{14}C]octanoate	240–230	10–32	109±14	200±40
	[1-^{14}C]palmitate	2800–4400	250–220	82±8	128±12

Table 2 Specific activities of peroxisomal enzyme markers in the course of the purification of high-density mitochondria from glucose-starved maize root tips

The specific activities of catalase, urate oxidase and acyl-CoA oxidase were determined in the crude extract, the crude mitochondria and the Percoll-purified high-density mitochondria. The acyl-CoA oxidase activity was measured in the presence of exogenous FAD with three different substrates. The results are means (±S.E.M.) of at least three experiments, except for urate oxidase, which was measured in one experiment; ND, not determined.

	Catalase (μmol/min per mg)	Urate oxidase (nmol/min per mg)	Acyl-CoA oxidase (nmol/min per mg)		
			Butyryl-CoA	Octanoyl-CoA	Palmitoyl-CoA
Crude extract	30±7	9.9	7±1	13±4	8.1±0.3
Crude mitochondria	41±8	4.3	6.3	1.4	ND
Percoll-purified mitochondria	3±1	< 0.3	< 1	< 1	< 1

Table 3 Production of acyl-CoA esters and organic acids after incubation of purified high-density mitochondria from glucose-starved maize root tips with [1-^{14}C]octanoate or [1-^{14}C]palmitate

High-density mitochondria from 1000 maize root tips after 48 h of glucose-starvation treatment were purified as described in the Materials and methods section. Approx. 140 μg protein of purified mitochondria was incubated with [1-^{14}C]octanoate or [1-^{14}C]palmitate for 40 min in medium M in the absence or in the presence of respiratory-chain inhibitors and in the presence and in the absence of carnitine. Incubations with hypo-osmotically damaged mitochondria were also carried out. The cocktail of respiratory-chain inhibitors consisted of 2 mM KCN, 1 mM NaN_3 plus 2 mM SHAM. This cocktail was added four times at 10 min intervals. The addition of the equivalent amount of dimethyl sulphoxide, in which SHAM was dissolved, had no effect. The radiolabelled products of these incubations were analysed by h.p.l.c. and radiometric measurement. No radiolabelled acetyl-CoA was detected, and the only significant peak of radioactivity in the fraction of acyl-CoA esters was the CoA ester of the radiolabelled precursor fatty acid. The major peaks of radioactivity in the organic acid fraction were citrate, malate and an organic acid probably derived from oxaloacetate. The production of radiolabelled organic acids was linear with time for 30 min and showed a small deviation from the initial rate between 30 and 40 min. The results of control incubations are means (±S.E.M.) from 5 independent experiments. The results of the incubations under modified conditions are means of the percentages of control from 2 (± range*) or at least 3 (±S.E.M.) independent experiments.

Incubation	Fatty acid	Production of acyl-CoA esters and organic acids (10^{-3} × d.p.m./mg of protein)	
		Fatty acid CoA ester	Organic acids
Control	[1-^{14}C]Octanoate	15±3	13±4
	[1-^{14}C]Palmitate	1200±400	14±5
		Production (% of control)	
		Fatty acid CoA ester	Organic acids
In the presence of respiratory-chain inhibitors	[1-^{14}C]Octanoate	225±20	11±6
	[1-^{14}C]Palmitate	90±15	21±6
With hypo-osmotically damaged mitochondria	[1-^{14}C]Octanoate	117±20	66±13
	[1-^{14}C]Palmitate	99±10	75±8
In the absence of carnitine	[1-^{14}C]Octanoate	136±23*	92±26
	[1-^{14}C]Palmitate	113±8*	104±7*

which is also consistent with previous reports on the purification of mitochondria from non-photosynthetic plant tissues (Neuburger et al., 1982; Journet, 1985; Douce et al., 1987). The analysis of contaminants was completed with the determination of peroxisomal enzyme activities in the course of the purification (Table 2). The purified mitochondria, whether during the experiments of Couée et al. (1992) or during the present experiments, showed variable levels of catalase activity ranging from 0 to 6 μmol/min per mg. When catalase activity was detectable, the specific activity in the purified mitochondria was 10-fold lower than that in the crude extract. However, a second washing of the crude mitochondrial pellet and further purification on a second Percoll density gradient did not remove the catalase activity, thus suggesting that it may be non-specifically adsorbed to mitochondrial material (see Journet, 1985). The activities of urate oxidase and acyl-CoA oxidase were not detectable with up to 150 μg of mitochondrial protein. Thus, the removal of peroxisomal enzyme markers during this purification was comparable with that obtained for highly purified mitochondria from potato tuber or cauliflower buds (Neuburger et al., 1982; Journet, 1985; Douce et al., 1987).

Table 3 shows the effects of respiratory-chain inhibitors on the production of acyl-CoA esters and of radiolabelled organic acids by glucose-starved mitochondria with octanoate or palmitate as fatty acid substrate. The mitochondria were incubated in medium M. The respiratory-chain inhibitors were added at regular intervals, as was done in the study of the peroxisome-containing fraction. Glucose-starved mitochondria were able to oxidize octanoate and palmitate. Radiolabelled citrate, isocitrate, succinate, fumarate, malate and a radiolabelled organic acid first putatively identified as oxaloacetate were observed by h.p.l.c. and radiometric analysis. Indeed, this putative oxaloacetate co-eluted only with intact oxaloacetate at 2.5 min and with no other organic acid of the tricarboxylic acid cycle. However, when subjected to the isolation procedure, oxaloacetate yielded two degradation products which were not recognized by malate

dehydrogenase (EC 1.1.1.37) under the assay conditions of Rej (1985). One product co-eluted with pyruvate, which is the product of β-decarboxylation (Rej, 1985), at 3.0 min. The other product, eluted at 2.5 min, could be derived from α-decarboxylation of oxaloacetate (Rej, 1985). The radiolabelled material eluted before 3.0 min may therefore correspond to degradation products of oxaloacetate. Citrate, the oxaloacetate-related products and malate represented respectively 60%, 20% and 10% of the total radioactivity of the organic acid fraction. No production of acetoacetate or β-hydroxybutyrate was observed, in contrast with mitochondrial β-oxidation in animals (see Schulz, 1991). Acyl-CoA esters were also analysed by h.p.l.c. No acetyl-CoA was detected, thus showing that any acetyl-CoA produced was a substrate for citrate synthase and yielded organic acids of the tricarboxylic acid cycle (see Douce, 1985).

The cocktail of respiratory-chain inhibitors containing cyanide, azide and SHAM gave an 80–90% inhibition of the production of all the radiolabelled organic acids, including the oxaloacetate-related products. Under these conditions of inhibition, no acetyl-CoA was detected (results not shown), although the levels of substrate fatty acyl-CoA esters were more than 90% of the control incubation (Table 3). When hypo-osmotically damaged mitochondria were used, the production of organic acids showed a decrease of at least 20% with no parallel changes in the production of the CoA esters of the substrate fatty acids.

The absence of exogenous carnitine in the incubation medium did not result in any decrease of β-oxidation-derived products (Table 3), which appeared to be in contradiction with the carnitine-dependent entry of acyl-CoA esters into animal mitochondria (see Schulz, 1991) and the carnitine-dependent respiration of palmitate and palmitoyl-CoA ester by plant mitochondria (Masterson et al., 1992). However, carnitine, which was determined as described in the Materials and methods section, was found to be present in the preparations of mitochondria at a level of 4 nmol/mg. Addition of the sample to the assay thus resulted in approx. 500 nM carnitine in the incubation medium. The kinetic parameters of carnitine acyltransferases for carnitine show a considerable range of variation with respect to assay conditions and to the physiological status of the tissue of origin (see Mills et al., 1984; Farrell et al., 1984; Fiol and Bieber, 1984; Bieber, 1988). With reasonable K_m and $V_{max.}$ values of 100 μM and 10 nmol/min per mg of mitochondrial protein, the presence of 500 nM carnitine under the conditions of the present assay would result in the production of 200 nM acylcarnitine in the course of the 40 min incubation. It could be calculated from the data in Table 3 that, in palmitate β-oxidation under control conditions, 2.5 μM palmitoyl-CoA ester was synthesized and 16.5 nM radiolabelled organic acids was produced. Thus the endogenous amount of carnitine would seem to be sufficient to sustain the transport of the necessary amount of palmitoyl-CoA esters.

High-density mitochondria, of apparent density 1.056 g/ml, were isolated from approx. 1000 non-starved maize root tips as described in the Materials and methods section. These preparations showed characteristics of purity, integrity and functionality similar to those of the mitochondria from glucose-starved tips, in agreement with the results of Couée et al. (1992). The specific activity of catalase was similar to that of glucose-starved mitochondria. When these mitochondria were incubated with radiolabelled fatty acid substrates in medium M, no radioactivity above the blank level was detected in the acetyl-CoA and organic acid fractions, whether with octanoate or palmitate. However, the levels of substrate fatty acyl-CoA esters were similar to those obtained with glucose-starved mitochondria

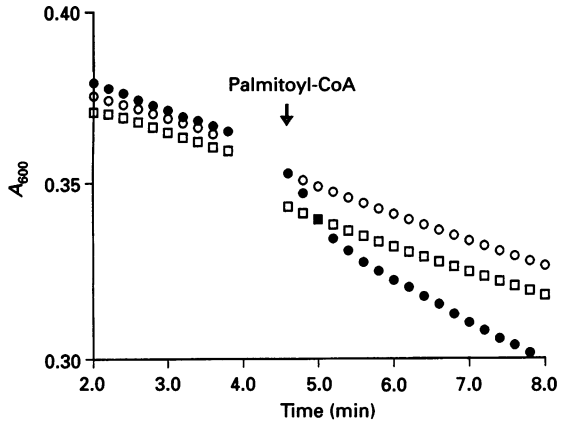

Figure 2 Acyl-CoA dehydrogenase activity in purified high-density mitochondria from glucose-starved maize root tips

The reduction of PMS/DCPIP was measured as described in the Materials and methods section in the absence of protein (□), or in the presence of 0.12 unit of acyl-CoA oxidase from *Candida* (○) or of 80 μg of purified high-density mitochondria from glucose-starved root tips (●) as catalyst in the presence of 50 μM palmitoyl-CoA. A typical experiment is shown. The arrow indicates the time of addition of 50 μM palmitoyl-CoA to the assay.

(results not shown). High-density mitochondria from non-starved root tips were therefore unable to carry out the β-oxidation of octanoate or palmitate.

Identification of acyl-CoA dehydrogenase activity

The ferricenium assay of acyl-CoA dehydrogenase activity (Lehman et al., 1990) was not used, because SHAM at millimolar concentrations gave a high background absorbance at 300 nm (ϵ 2100 $M^{-1} \cdot cm^{-1}$). The PMS/DCPIP assay was therefore chosen for its reportedly high sensitivity (Lehman et al., 1990). The blank rate was the same in the absence or in the presence of mitochondrial samples and was not decreased by the presence of *N*-ethylmaleimide (NEM). Furthermore, for the purpose of identifying the activity, NEM was not used, as thiol reagents may have inhibitory effects if the enzyme is not saturated with FAD (see Okamura-Ikeda et al., 1985). Samples of purified high-density mitochondria from glucose-starved maize root tips gave a significant substrate-specific decrease in absorbance (Figure 2), thus suggesting the presence of acyl-CoA dehydrogenase activity (see Lehman et al., 1990; Izai et al., 1992). This activity was obtained with butyryl-CoA, octanoyl-CoA and palmitoyl-CoA as substrates (Table 4). The decrease in A_{600} could have been driven by acyl-CoA oxidase activities. However, even in the presence of 50 μM exogenous FAD, the activity of contaminating acyl-CoA oxidase in purified mitochondria was not detectable. Furthermore, 2 μl portions of a commercial preparation of acyl-CoA oxidase from *Candida* gave no significant substrate-specific decrease in absorbance in the presence of octanoyl-CoA or palmitoyl-CoA (Figure 2), although this preparation showed a 60 units/ml catalytic concentration of H_2O_2 production with palmitoyl-CoA as substrate in the buffer conditions (minus SHAM) of the PMS/DCPIP assay.

The observed decrease in absorbance could also have been due to acyl-CoA esterase activity effecting the release of CoASH from acyl-CoA substrates and the subsequent reduction of PMS and DCPIP (Lehman et al., 1990). Indeed, desalted crude extracts and purified mitochondria showed activity catalysing the acyl-CoA-dependent reduction of DTNB (Table 4). However, in

Table 4 Specific activities of DTNB reduction and PMS/DCPIP reduction in crude extracts and purified high-density mitochondria from glucose-starved maize root tips

Crude extracts and purified mitochondria were obtained from approx. 1000 glucose-starved maize root tips as described in the Materials and methods section. The crude extracts were desalted by gel-filtration on Econo-pac (BioRad) desalting columns in order to remove low-molecular-mass reductants. The activities of DTNB reduction and PMS/DCPIP reduction were determined in the presence of butyryl-CoA, octanoyl-CoA or palmitoyl-CoA as substrate, as described in the Materials and methods section. The results are expressed as nmol of TNB^{2-} or reduced DCPIP produced/min per mg of protein. The results are means of 2 (±range*) or at least 3 (±S.E.M.) experiments; ND, not determined.

Substrate	Fraction	DTNB-reducing specific activity (nmol/min per mg)	PMS/DCPIP-reducing specific activity (nmol/min per mg)
Butyryl-CoA	Crude extract	ND	ND
	Mitochondria	1.3±0.1*	2.5±0.4*
Octanoyl-CoA	Crude extract	2.03±0.03*	0.1±0.1*
	Mitochondria	1.0±0.5	4±2
Palmitoyl-CoA	Crude extract	4.1±0.2	1.2±0.2
	Mitochondria	2±1	5±1.5

Table 5 Comparison of the activities of DTNB reduction, PMS/DCPIP reduction and CoASH production in purified high-density mitochondria from glucose-starved maize root tips

The activities of DTNB reduction and PMS/DCPIP reduction were determined as described in the Materials and methods section with 170 μg of mitochondrial protein in the assay mixture (1 ml) and 50 μM palmitoyl-CoA as substrate. Blanks in the absence of mitochondrial protein gave no significant rates in either assay. An example where the DTNB-reducing activity was the highest is given. At the end of a 5 min incubation period in the PMS/DCPIP assay, the medium was treated as described in the Materials and methods section and the resulting fraction was analysed by h.p.l.c. (Figure 3). The results are expressed in nmol of the specified products obtained at the end of the incubation. These amounts were determined from the h.p.l.c. profiles and the recordings of the spectrophotometric assays, to take into account the 1 min lag phase and the small burst of respectively the DTNB assay and the PMS/DCPIP assay. The production of CoASH and CoAS-CoA is expressed in equivalents of CoASH.

Measurement	Production of:	Amount (nmol)
DTNB reduction	TNB^{2-}	2.8
PMS/DCPIP reduction	Reduced DCPIP	4.2
H.p.l.c. analysis	Equivalent CoASH	0.5

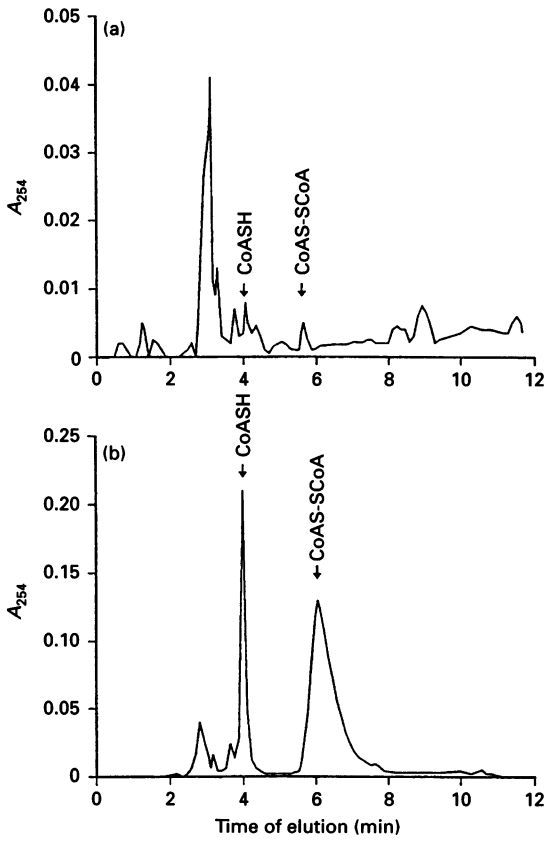

Figure 3 Acyl-CoA esterase activity in purified high-density mitochondria from glucose-starved maize root tips

Purified high-density mitochondria from glucose-starved root tips were incubated for 5 min in the presence of 50 μM palmitoyl-CoA under the conditions of the PMS/DCPIP assay. The incubation medium was then treated through the isolation procedure for acyl-CoA esters, which gives a recovery of CoA, whether reduced or oxidized, of over 99%. The resulting fraction was analysed by h.p.l.c. as described in the Materials and methods section. The elution profiles of such a fraction (**a**) and that of the same fraction with CoASH and CoAS-SCoA as internal standards (**b**) are shown.

purified mitochondria, the activity of PMS/DCPIP reduction was significantly higher than that of DTNB reduction (Table 4). We verified by h.p.l.c. that the non-enzymic reduction of PMS/DCPIP by CoASH yielded CoAS-SCoA. Given the 1:2 stoichiometry of the reaction between the DCPIP/$DCPIPH_2$ and the CoAS-SCoA/CoASH redox couples, the CoASH production catalysed by acyl-CoA esterase would give a 2-fold lower rate of DCPIP reduction. It thus appeared that PMS/DCPIP reduction by purified mitochondria did not reflect acyl-CoA esterase activity, unless this activity was activated in the presence of PMS and DCPIP. However, this latter possibility seemed unlikely, since, with desalted crude extracts, the rate of PMS/DCPIP reduction was less than 25% of the DTNB-reducing activity (Table 4), which was lower than the 2-fold factor due to stoichiometry and thus suggested that acyl-CoA esterases were poorly active under the conditions of the PMS/DCPIP assay.

This was verified by carrying out an h.p.l.c. analysis of CoASH and CoAS-SCoA in parallel with the PMS/DCPIP reducing assay. The elution of CoASH and CoAS-SCoA when added exogenously to the PMS/DCPIP assay is shown in Figure 3. Both reduced and oxidized CoA were recovered with a yield higher than 99% through the isolation procedures before h.p.l.c. analysis. Figure 3 also shows the h.p.l.c. profile of the compounds present in the PMS/DCPIP assay at the end of the reaction in the presence of purified mitochondria with palmitoyl-CoA as substrate. The two peaks that could be identified as CoASH and CoAS-SCoA appeared in the course of the incubation, whereas the rest of the profile was identical with the profile at the beginning of the reaction. Quantification of the h.p.l.c. analyses (Table 5) shows that the release of CoASH and CoAS-SCoA during the PMS/DCPIP assay was negligible in comparison with the production of reduced DCPIP. It was also negligible relative to the production of TNB^{2-} in the DTNB assay, thus suggesting, like the results obtained with desalted crude extracts, that acyl-CoA esterase activity was poorly active in the PMS/DCPIP assay. The reduction of PMS/DCPIP by purified mitochondria could therefore be ascribed to acyl-CoA dehydrogenase activity. Furthermore, whereas acyl-CoA esterase specific activity decreased, the PMS/DCPIP-reducing specific activity increased in the course of the purification from the crude extract to the mitochondria.

Table 6 Estimation of the β-oxidation capacities of peroxisomes and mitochondria in 1000 glucose-starved maize root tips

The β-oxidation capacity of peroxisomes in the root tip was calculated from the activity of the peroxisome-containing fraction from 1000 root tips, in the peroxisome-facilitating medium P (Table 1), and from the yield of peroxisomes, which was estimated from the yield of 3-hydroxyacyl-CoA dehydrogenase activity (32 ± 1%, mean ± S.E.M.). The β-oxidation capacity of mitochondria in the root tip was calculated from the activity of the purified high-density mitochondria from 1000 root tips, in the mitochondria-facilitating medium M (Table 2), and from the yield of mitochondria in the preparation, which was estimated from the yield of fumarase activity (11 ± 2%, mean ± S.E.M.). The yields were calculated from the means of at least three experiments, relative to the total tissue contents, as determined by Brouquisse et al. (1991). The production of β-oxidation end-products was linear with time for at least 40 min for peroxisomal β-oxidation (Table 1) and for 30 min for mitochondrial β-oxidation (Table 3). In this latter case, the mean rate of production over 40 min showed a 10% deviation relative to the initial velocity over the period of linearity. The amounts of radiolabelled products obtained per incubation were therefore transformed into initial velocities expressed in pmol/min by using the specific radioactivities of the label given in the Materials and methods section. The values of β-oxidation are the minimum and maximum activities that were obtained in the course of the experiments.

		Initial velocity (pmol/min)	
		Octanoate	Palmitate
Mitochondria	Purified mitochondria	4–10	2–7
	1000 root tips	40–100	20–70
Peroxisomes	Peroxisome-containing fraction	45–157	1200–1370
	1000 root tips	150–500	3800–4300

Relative contributions of mitochondrial and peroxisomal β-oxidation in glucose-starved maize root tips

The β-oxidation capacity of the peroxisomal pool in the glucose-starved root tip was calculated from the β-oxidation activity in the peroxisome-containing fraction in medium P, which facilitated peroxisomal β-oxidation, and the recoveries of fumarase and 3-hydroxyacyl-CoA dehydrogenase in this fraction. This β-oxidation activity was not inhibited by respiratory-chain inhibitors (Table 1), and could therefore be ascribed to peroxisomes. The activity of 3-hydroxyacyl-CoA dehydrogenase was probably distributed between the peroxisomal and mitochondrial compartments, given that both mitochondrial and peroxisomal β-oxidations were active. However, the activity of 3-hydroxyacyl-CoA dehydrogenase in purified high-density mitochondria could account for less than 10% of the activity in the peroxisome-containing fraction. Furthermore, the yield of 3-hydroxyacyl-CoA dehydrogenase activity in the peroxisome-containing fraction (32 ± 1%, mean ± S.E.M.) was equal to the yield of fumarase activity (31 ± 3%, mean ± S.E.M.). The yield of peroxisomes was therefore equal to the yield of 3-hydroxyacyl-CoA dehydrogenase activity. The β-oxidation capacity of the mitochondrial pool in the root tip was calculated from the β-oxidation activity in the purified mitochondria in medium M, which facilitated mitochondrial β-oxidation, and the yield of fumarase activity in this preparation. This β-oxidation activity could be inhibited 5–10-fold by respiratory-chain inhibitors (Table 3) and was therefore ascribed mostly to mitochondria. The resulting estimation was not corrected for the possible decarboxylation of oxaloacetate which has been described above, as the 20% correction was negligible for the comparison with peroxisomal β-oxidation. Table 6 shows that the capacity of palmitate β-oxidation in glucose-starved root tips was 60–200-fold higher in peroxisomes than in mitochondria. In contrast, the capacity of octanoate β-oxidation was only 1.5–12-fold higher in peroxisomes than in mitochondria. These measurements of capacity could, however, be criticized, as the conditions of incubation may not have been optimal.

DISCUSSION

The activity of β-oxidation detected in highly purified mitochondria from glucose-starved maize root tips presented a number of distinctive characteristics which showed that it could not be ascribed to contaminating peroxisomal enzymes. It was almost completely inhibited by the cocktail of cyanide, azide and SHAM (Table 3), in contrast with peroxisomal β-oxidation (Table 1). The inhibitors only affected the production of radiolabelled organic acids. The production of substrate acyl-CoA esters was not inhibited and acetyl-CoA did not accumulate, thus showing that the β-oxidation activity, rather than the acyl-CoA synthetase or the citrate synthase activities, was inhibited. In contrast, in the peroxisome-containing fraction, when assayed in the medium facilitating peroxisomal β-oxidation, the cocktail of respiratory-chain inhibitors had an activating effect on the production of acetyl-CoA (Table 1). Disruption of mitochondria by hypo-osmotic conditions also resulted in a partial inhibition of mitochondrial β-oxidation, in accordance with the work of Sumegi et al. (1991) showing that, in gently disrupted rat liver mitochondria, the respiration-linked oxidation of saturated fatty acyl-CoA esters was decreased by 50%. Glucose starvation had an activating effect on the β-oxidation activities in both mitochondrial and peroxisome-containing preparations, in agreement with the shift from carbohydrate catabolism to fatty acid and protein utilization (Brouquisse et al., 1991, 1992; Dieuaide et al., 1992). Control experiments with excised maize root tips incubated in the presence of 0.2 M glucose showed that the inactivation of glycolytic and mitochondrial enzymes (Brouquisse et al., 1991), the activation of β-oxidation (Dieuaide et al., 1992) and the modifications of the mitochondrial pools (Couée et al., 1992) could be ascribed to glucose starvation and not to excision wounding. This activating effect showed some specificity of substrate, because in peroxisomes the β-oxidation of palmitate, but not that of octanoate, was increased (Table 1). In contrast, in mitochondria the β-oxidation of both palmitate and octanoate was increased (Table 3). Finally, the enzyme activity which is characteristic of mitochondrial β-oxidation in mammalian cells (see Schulz, 1991), acyl-CoA dehydrogenase, was shown to be associated with mitochondria from glucose-starved maize root tips (Tables 4 and 5).

The distribution of β-oxidation fluxes between mitochondria and peroxisomes, in better-described material such as hepatocytes, is still a matter of controversy (see Rognstad, 1991). Labelling experiments *in vivo* will be required to study this distribution in maize root tips. Nevertheless, we attempted from our results to estimate the capacities of peroxisomes and mitochondria in a root tip to carry out β-oxidation (Table 6). In glucose-fed root tips, the peroxisomal β-oxidation (Table 1) appeared to be effective on long-chain fatty acids such as palmitate, in accordance with previous results (Gerhardt, 1986; Dieuaide et al., 1992), and on medium-chain fatty acids such as octanoate. In isolated peroxisomes, the production of acetyl-CoA from exogenous octanoate was at the lowest 30% of that from exogenous palmitate (Table 1). Thus it appeared that, in glucose-fed root tips, β-oxidation could be carried out from palmitate down to short-chain fatty acids solely by peroxisomes. In contrast, under the effects of glucose starvation, β-oxidation could be carried out by both peroxisomes and mitochondria, and it thus seemed that potential co-operation could take place between the two compartments. Palmitate β-oxidation by peroxisomes showed a 4–12-fold increase after 48 h of glucose

starvation, which was higher than the 2–5-fold increase previously described after 24 h of starvation treatment (Dieuaide et al., 1992). In contrast, octanoate β-oxidation by peroxisomes did not show any increase (Table 1). It therefore appears that, under the conditions of glucose starvation, the discrepancy between palmitate and octanoate β-oxidation may result in a badly integrated total β-oxidation in peroxisomes. The mitochondrial capacity to oxidize palmitate was considerably lower in comparison with that obtained with peroxisomes (Table 6), thus raising the question of its quantitative importance *in vivo*. However, the β-oxidation of octanoate by mitochondria was not negligible in comparison with that of peroxisomes (Table 6), thus suggesting some role for mitochondria in the oxidation of medium-chain fatty acids, and therefore in the complete β-oxidation of fatty acids, as has been described in mammalian cells (see Schulz, 1991). Mitochondrial β-oxidation may be thought to function in situations of spill-over from the peroxisomes, as may happen in situations of glucose starvation with the increased availability of fatty acid substrates (Dorne et al., 1987; Brouquisse et al., 1991). The absence of a detectable β-oxidation activity in mitochondria from non-starved tissue may explain why mitochondrial β-oxidation has remained relatively un-noticed in higher plant cells. The mechanisms of its activation during glucose starvation remain to be elucidated and are under investigation in our laboratory.

REFERENCES

Aebi, H. E. (1987) in Methods of Enzymatic Analysis, volume 3 (Bergmeyer, H. U., Bergmeyer, J. and Grassl, M., eds.), pp. 273–286, VCH, Weinheim

ap Rees, T. (1990) in Plant Physiology, Biochemistry and Molecular Biology (Dennis, D. T. and Turpin, D. H., eds.), pp. 106–123, Longman Scientific and Technical, Harlow

Baker, F. C. and Schooley, D. A. (1979) Anal. Biochem. **94**, 417–424

Bieber, L. L. (1988) Annu. Rev. Biochem. **57**, 261–283

Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254

Brouquisse, R., James, F., Raymond, P. and Pradet, A. (1991) Plant Physiol. **96**, 619–626

Brouquisse, R., James, F., Pradet, A. and Raymond, P. (1992) Planta **188**, 384–395

Couée, I., Jan, M., Carde, J.-P., Brouquisse, R., Raymond, P. and Pradet, A. (1992) Plant Physiol. **98**, 411–421

Day, D. A., Neuburger, M. and Douce, R. (1984) Arch. Biochem. Biophys. **231**, 233–242

Dieuaide, M., Brouquisse, R., Pradet, A. and Raymond, P. (1992) Plant Physiol. **99**, 595–600

Dorne, A. J., Bligny, R., Rebeillé, F., Roby, C. and Douce, R. (1987) Plant Physiol. Biochem. **25**, 589–595

Douce, R. (1985) Mitochondria in Higher Plants: Structure, Function and Biogenesis, Academic Press, New York

Douce, R., Bourguignon, J., Brouquisse, R. and Neuburger, M. (1987) Methods Enzymol. **148**, 403–415

Engel, P. C. (1992) in Chemistry and Biochemistry of Flavoenzymes, volume 3 (Müller, F., ed.), pp. 597–655, CRC Press, Boca Raton, Ann Arbor and London

Farrell, S. O., Fiol, C. J., Reddy, J. K. and Bieber, L. L. (1984) J. Biol. Chem. **259**, 13089–13095

Fiol, C. J. and Bieber, L. L. (1984) J. Biol. Chem. **259**, 13084–13088

Gerhardt, B. (1986) Physiol. Vég. **24**, 397–410

Gerhardt, B. (1987) Methods Enzymol. **148**, 516–525

Harwood, J. L. (1988) Annu. Rev. Plant Physiol. **39**, 101–138

Hill, R. L. and Bradshaw, R. A. (1969) Methods Enzymol. **13**, 91–99

Hong, Y.-N. and Schopfer, P. (1981) Planta **152**, 325–335

Hosokawa, Y., Shimomura, Y., Harris, R. A. and Osawa, T. (1986) Anal. Biochem. **153**, 45–49

Huang, A. H. C., Trelease, R. N. and Moore, T. S., Jr. (1983) Plant Peroxisomes, Academic Press, New York

Izai, K., Uchida, Y., Orii, T., Yamamoto, S. and Hashimoto, T. (1992) J. Biol. Chem. **267**, 1027–1033

Journet, E. (1985) Thèse de Doctorat es Sciences Naturelles, Université Scientifique et Médicale de Grenoble

Lehman, T. C., Hale, D. E., Bhala, A. and Thorpe, C. (1990) Anal. Biochem. **186**, 280–284

Macri, F., Vianello, A., Braidot, E. and Zancani, M. (1991) Biochim. Biophys. Acta **1058**, 249–255

Mannaerts, G. P., Debeer, L. J., Thomas, J. and De Schepper, P. J. (1979) J. Biol. Chem. **254**, 4585–4595

Masterson, C., Wood, C. and Thomas, D. R. (1992) Plant Cell Environ. **15**, 313–320

McGarry, J. D. and Foster, D. W. (1985) in Methods of Enzymatic Analysis, volume 8 (Bergmeyer, H. U., Bergmeyer, J. and Grassl, M., eds.), pp. 474–481, VCH, Weinheim

Miernyk, J. A., Thomas, D. R. and Wood, C. (1991) Plant Physiol. **95**, 564–569

Mills, S. E., Foster, D. W. and McGarry, J. D. (1984) Biochem. J. **219**, 601–608

Neuburger, M., Journet, E.-P., Bligny, R., Carde, J.-P. and Douce, R. (1982) Arch. Biochem. Biophys. **217**, 312–323

Okamura-Ikeda, K., Ikeda, Y. and Tanaka, K. (1985) J. Biol. Chem. **260**, 1338–1345

Osmundsen, H., Bremer, J. and Pederson, J. I. (1991) Biochim. Biophys. Acta **1085**, 141–158

Panter, R. A. and Mudd, J. B. (1973) Biochem. J. **134**, 655–658

Raymond, P., Spiteri, A., Dieuaide, M., Gerhardt, B. and Pradet, A. (1992) Plant Physiol. Biochem. **30**, 153–161

Rej, R. (1985) in Methods of Enzymatic Analysis, volume 7 (Bergmeyer, H. U., Bergmeyer, J. and Grassl, M., eds.), pp. 59–67, VCH, Weinheim

Riddles, P. W., Blakeley, R. L. and Zerner, B. (1983) Methods Enzymol. **91**, 49–60

Rognstad, R. (1991) Biochem. J. **279**, 147–150

Sabbagh, E., Cuebas, D. and Schulz, H. (1985) J. Biol. Chem. **260**, 7337–7342

Saglio, P. and Pradet, A. (1980) Plant Physiol. **66**, 516–519

Saglio, P., Rancillac, M., Bruzeau, F. and Pradet, A. (1984) Plant Physiol. **76**, 151–154

Salon, C., Raymond, P. and Pradet, A. (1988) J. Biol. Chem. **263**, 12278–12287

Schulz, H. (1991) Biochim. Biophys. Acta **1081**, 109–120

Sumegi, B., Porpagy, Z. and Alkonyi, I. (1991) Biochim. Biophys. Acta **1081**, 121–128

Wood, C., Jalil, M. N. H., McLaren, I., Yong, B. C. S., Ariffin, A., McNeil, P. H., Burgess, N. and Thomas, D. R. (1984) Planta **161**, 255–260

Wood, C., Burgess, N. and Thomas, D. R. (1986) Planta **167**, 54–57

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