Mitochondrial and peroxisomal β -oxidation capacities of organs from a non-oilseed plant

Christine Masterson* and Clifford Wood

Department of Biological and Nutritional Sciences, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, UK

Until recently, β -oxidation was believed to be exclusively located in the peroxisomes of all higher plants. Whilst this is true for germinating oilseeds undergoing gluconeogenesis, evidence demonstrating mitochondrial β -oxidation in other plant systems has refuted this central dogma of plant lipid metabolism. This report describes a comparative study of the dual mitochondrial and peroxisomal β -oxidation capacities of plant organs. Oxidation of [$1\text{-}^{14}\text{C}$]palmitate was measured in the cotyledons, plumules and radicles of *Pisum sativum* L., which is a starchy seed, over a 14 day period from the commencement of imbibition. Respiratory chain inhibitors were used for differentiating between mitochondrial and peroxisomal β -oxidation. Peroxisomal β -oxidation gave a steady, baseline rate and, in the early stages of seedling development, accounted for 70–100% of the β -oxidation observed. Mitochondrial β -oxidation gave peaks of activity at days 7 and 10–11, accounting for up to 82% of the total β -oxidation activity at these times. These peaks coincide with key stages of seedling development and were not observed when normal development was disrupted by growth in the dark. Peroxisomal β -oxidation was unaffected by etiolation. Since mitochondrial β -oxidation was overt only during times of intense biosynthetic activity it might be switched on or off during seedling development. In contrast, peroxisomes maintained a continuous, low β -oxidation activity that could be essential in removing harmful free fatty acids, e.g. those produced by protein and lipid turnover.

Keywords: *Pisum sativum*; β -oxidation; mitochondria; peroxisomes; non-oilseed plant; seedling development

1. INTRODUCTION

A central dogma of plant lipid metabolism has been that β -oxidation occurs exclusively within the peroxisomes/glyoxysomes of all higher plant tissues (Gerhardt 1992; Kindl 1993). Whilst this is true for germinating oilseeds undergoing gluconeogenesis, the overwhelming accumulation of evidence over the years demonstrating mitochondrial β -oxidation is now finally forcing an acceptance of a dual location for β -oxidation in higher plants (Masterson & Wood 2000a).

One of the characteristics that differentiates mitochondrial from peroxisomal β -oxidation in higher plants is the first enzyme in the four-enzyme β -oxidation spiral (Schulz 1991). The first enzyme in peroxisomes is acyl CoA oxidase (EC 1.3.3.6), which is a flavin–adenine dinucleotide (FAD)-containing enzyme that is reoxidized by direct transfer of electrons from reduced flavin–adenine dinucleotide (FADH₂) to O₂ in order to form H₂O₂, which is subsequently broken down by catalase. In contrast, mitochondria possess acyl CoA dehydrogenases (EC 1.3.99.2,3), which are also FAD-containing enzymes, but the FADH₂ in this case is reoxidized by the electron transport chain. Masterson *et al.* (2000) measured acyl CoA dehydrogenase activity in developing pea seedlings using butyryl, lauryl and palmitoyl CoA as substrates, and found pronounced peaks of activity that corresponded to specific developmental stages. A preliminary attempt at distinguishing mitochondrial β -oxidation from peroxisomal β -oxidation in whole seedlings (Masterson & Wood 2000b) showed that mitochondrial β -oxidation peaked at the same developmental stages as the acyl CoA dehydrogenase activities.

The identification of a second distinct and independent pathway from that in the peroxisomes has shown that the plant β -oxidation system itself, its roles and its regulation are not completely understood. Historically, β -oxidation has mainly been studied in germinating oilseeds, where stored triacylglycerols are channelled into gluconeogenesis within the glyoxysomes. However, β -oxidation has other functions in plant metabolism (Gerhardt 1992). It provides respiratory substrates for complete oxidation to carbon dioxide and water thereby leading to the generation of adenosine triphosphate (ATP), it provides acetyl CoA for biosynthesis, it modifies acyl CoAs to produce metabolically important compounds, and it removes harmful free fatty acids originating from both protein and membrane lipid turnover. The subcellular location of β -oxidation in all of these physiological processes is not known. There are two obvious questions regarding plant β -oxidation that require immediate answer.

- (i) What is the physiological role of two β -oxidation sites?
- (ii) What is the flux to these two sites?

To the authors' knowledge, this report is the first to address these two questions by direct measurement of the dual mitochondrial and peroxisomal β -oxidation capacities of plant organs, namely the cotyledons, plumules and radicles of *Pisum sativum*, during early seedling development.

2. MATERIAL AND METHODS

(a) *Material*

General acids, bases and inorganic salts (AnalaR grade where possible) were supplied by BDH Chemicals (Poole,

*Author for correspondence (christine.masterson@ncl.ac.uk).

Dorset, UK). All organic buffers, osmotica, bovine serum albumin (BSA), adenosine diphosphate (ADP), ATP, thiamine pyrophosphate, nicotinamide adenine dinucleotide (NAD^+), coenzyme A (CoASH), L-carnitine and salicylhydroxamic acid (SHAM) were obtained from Sigma (London) Chemical Co. (Poole, Dorset, UK). Bio-Rad protein assay dye reagent was obtained from Bio-Rad Laboratories Ltd (Hemel Hempstead, Hertfordshire, UK). [$1\text{-}^{14}\text{C}$]Palmitic acid was obtained from Amersham (Little Chalfont, Buckinghamshire, UK). Pea seeds were supplied by Batchelor Foods Ltd (Worksop, Nottinghamshire, UK) and castor oil seeds were from Herbiseed (Wokingham, Berkshire, UK).

(b) *Growth of plants*

The pea seeds (*P. sativum* L. cv. Bunting) were surface sterilized in 6% (w/v) sodium hypochlorite for 5 min, thoroughly rinsed and then imbibed in running tap water for 8 h. The seeds were then germinated on damp tissue paper at 25 °C for a further 64 h in the dark until the radicles were *ca.* 3 cm long and the plumules were just apparent. The germinated seedlings were then grown for up to 14 days by suspension over trays of water at room temperature in either natural daylight (light grown) or in the dark (dark grown), with frequent water changes.

Castor oil seeds (*Ricinus communis* L. cv. Gibsonii) were imbibed in running tap water for 8 h and germinated in moist vermiculite at 30 °C for 5 days in the dark.

(c) *Assays*

Total palmitate oxidation rates for whole organ homogenates were calculated from the sum of $^{14}\text{CO}_2$ and ^{14}C -labelled perchloric acid-soluble products, which were produced from [$1\text{-}^{14}\text{C}$]palmitic acid in the presence of all necessary cofactors (Piot *et al.* 1998).

Ten whole seedlings were separated into individual organs, namely the endosperm of the castor oil seeds and the cotyledons (minus testa), radicles and plumules of the pea seeds. The organs were weighed and homogenized in the minimum required volume of a medium comprising 200 mM sucrose, 20 mM Tris-HCl pH 7.5, 4 mM KH_2PO_4 and 1 mM MgSO_4 . Palmitate oxidation was measured in small glass flasks in a total volume of 0.5 ml containing 0.2 ml tissue homogenate. The final assay mix contained 200 mM sucrose, 20 mM Tris-HCl pH 7.5, 4 mM KH_2PO_4 , 1 mM MgSO_4 , 0.2 mM ADP, 0.17 mM thiamine pyrophosphate, 0.23 mM NAD^+ , 1 mM ATP, 0.01 mM CoASH, 0.05 mM L-malate and 1 mM L-carnitine. Peroxisomal palmitate oxidation was determined in the presence of a cocktail of respiratory chain inhibitors comprising 2 mM potassium cyanide, 1 mM sodium azide and 2 mM SHAM. Flasks were pre-incubated for 5 min at 28 °C before the addition of 0.1 ml of substrate consisting of 0.15 mM palmitic acid bound to BSA in a 5:1 molar ratio containing 0.2 μCi (7.4 kBq) [$1\text{-}^{14}\text{C}$]palmitic acid (specific activity 50 mCi (1.85 GBq) mmol^{-1}). Incubation was carried out for 30 min at 28 °C with agitation and terminated with 0.2 ml of 3 M perchloric acid. The $^{14}\text{CO}_2$ that was released was trapped in 0.2 ml of 10% (w/v) KOH and measured by liquid scintillation counting. After 90 min at 4 °C, the acidified incubation mixture was centrifuged at 10 000 *g* for 5 min and 0.2 ml of the supernatant containing ^{14}C -labelled perchloric acid-soluble products was also assayed for radioactivity by liquid scintillation counting. The total and peroxisomal palmitate oxidation rates were calculated from the sum of the $^{14}\text{CO}_2$ and ^{14}C -labelled perchloric acid-soluble products that were formed in the absence and presence of respiratory chain inhibitors,

respectively. The mitochondrial oxidation rates were calculated as the difference between the total and peroxisomal oxidation rates.

Assays were performed every 24 h from the commencement of imbibition for the pea organs and at 5 days old for the castor oil seed endosperm. Fresh:dry weight ratios were found by drying the organs to constant weight at 110 °C and protein was measured spectrophotometrically using the protein dye Coomassie blue (Bradford 1976) with BSA as the protein standard.

3. RESULTS AND DISCUSSION

β -Oxidation was measured radiochemically in homogenates of the plant organs that contained both mitochondria and peroxisomes. The total oxidation of [$1\text{-}^{14}\text{C}$]palmitate was calculated from the sum of the $^{14}\text{CO}_2$ and ^{14}C -labelled perchloric acid-soluble products that were produced in the presence of all necessary cofactors. Peroxisomal palmitate oxidation was determined by including a cocktail of respiratory chain inhibitors in the assay medium, which curtailed mitochondrial β -oxidation whilst having no inhibitory effect on the peroxisomal β -oxidation rates. The mitochondrial oxidation rates were calculated as the difference between the total and peroxisomal rates.

This method is possible because of differences in the first enzyme in the β -oxidation spiral at the two sites (Schulz 1991). The first enzyme in mitochondria is acyl CoA dehydrogenase (EC 1.3.99.2,3), which is an FAD-containing enzyme. The FADH_2 resulting from oxidation of the acyl CoA substrates is reoxidized by the mitochondrial electron transport chain. Thus, inhibitors of the electron transport chain inhibit mitochondrial β -oxidation. The first enzyme of peroxisomal β -oxidation is acyl CoA oxidase (EC 1.3.3.6), which is also an FAD-containing enzyme. However, the FADH_2 produced here is reoxidized by direct transfer of electrons to O_2 , which forms H_2O_2 . Consequently, peroxisomal β -oxidation is not inhibited by respiratory chain inhibitors. The cocktail of inhibitors used contained potassium cyanide and sodium azide for inhibiting the cyanide-sensitive cytochrome oxidase and SHAM for inhibiting the cyanide-resistant alternative oxidase found in plant mitochondria (Douce 1985). This cocktail is known to inhibit completely all mitochondrial electron transport chain activity in plants at the concentrations used.

Initially, it was vital to show that these inhibitors did not affect peroxisomal β -oxidation. The efficacy of the method was therefore first tested under conditions where β -oxidation is known to be exclusively peroxisomal. Five-day-old castor oil seed (*R. communis* L.) endosperm is exclusively gluconeogenic and β -oxidation occurs within specialized peroxisomes called glyoxysomes (Cooper & Beevers 1969). The endosperm gave a β -oxidation rate of 58 ± 9 pmol palmitate oxidized per minute per gram of dry weight in the absence of the inhibitor cocktail. This rate was not reduced in the presence of the inhibitors. This verifies that neither the cytochrome pathway nor the alternative oxidase is involved in the oxidation of palmitate and that the inhibitors have no effect upon peroxisomal β -oxidation. The method is therefore suitable for distinguishing between the mitochondrial and peroxi-

somal β -oxidation rates in *P. sativum*, which is a starchy seed that is non-gluconeogenic.

β -Oxidation was measured in the cotyledons, plumules and radicles of *P. sativum* over a 14 day period, from the commencement of imbibition by the dry pea seeds through germination, emergence and the early stages of vegetative growth leading to the establishment of independent seedlings with leaves fully unfolded up to the second node. The dry weight and protein content of the radicles and plumules increased over this time-period whilst those of the cotyledons decreased as they fuelled the growth of the embryos. β -Oxidation is therefore expressed per seedling or per organ in figure 1 as this most clearly describes the variation in rates with development.

The total β -oxidation in the seedling varied over the 14 days post-imbibition (figure 1a). The β -oxidation rates were lowest, at 13–20 pmol palmitate oxidized per minute per seedling, over the first 3 days of germination as the dry seed became fully imbibed, the radicle became emergent and the plumule became apparent. Over the next 4 days, the β -oxidation rates increased, culminating in a peak of 160 pmol palmitate oxidized per minute per seedling at day 7, which was the highest β -oxidation rate observed over the 14 day period. By day 9, the β -oxidation rates had fallen again to 40 pmol palmitate oxidized per minute per seedling. This 6 day period from day 4 to day 9 showed massive development of the plumules and radicles, which entered the log phase of their growth curves at day 6. The β -oxidation rates rose again to a peak of 75 pmol palmitate oxidized per minute per seedling at day 10 before falling gradually back to a rate of 40 pmol palmitate oxidized per minute per seedling by day 14. By days 10 and 11 the first pair of leaflets was fully unfolded at the first node and seedling development was independent of the cotyledon. In addition, secondary radicles were developing as the root system matured.

The cotyledon was the site of the greatest β -oxidation activity over the first 8 days, contributing 50–90% of the total seedling β -oxidation (compare figure 1a,d). The cotyledon's contribution to the total seedling β -oxidation fell to 30% during days 9–14, whilst the radicles contributed 40–50% of the total and the plumules 20–30% (compare figure 1a,d,g,j). The cotyledons, which occupied 40–98% of the seedling dry weight and contributed 30–90% of the total seedling β -oxidation over the 14 days post-imbibition, are clearly the prime site of β -oxidation in developing pea seedlings. The contributions of the mitochondria and peroxisomes to the total palmitate oxidation are compared in figure 1.

The total β -oxidation rates in the cotyledon followed a similar pattern to the total seedling β -oxidation rates over the 14 days, exhibiting low rates (7–9 pmol palmitate oxidized per minute per cotyledon) during days 1–3 and rising to a peak of 141 pmol palmitate oxidized per minute per cotyledon at day 7 before falling back to background levels at day 9, followed by a smaller peak of 28 pmol palmitate oxidized per minute per cotyledon at days 10 and 11 (figure 1d). The peroxisomal β -oxidation rates predominated over the first 2 days of germination, accounting for 90–100% of the β -oxidation at this time (figure 1e). These rates did not fluctuate greatly over the 14 days, remaining at *ca.* 7 pmol palmitate oxidized per

minute per cotyledon. Rather, the peak at day 7 was due to a 24-fold increase in the mitochondrial β -oxidation rates (figure 1f), with mitochondrial β -oxidation accounting for 82% of the oxidation rate observed. Days 4–9 was the time-period when the plumule was developing greatly. By day 4 the epicotyl had elongated to 1.5 cm and greening of the plumule was observed at day 5. Over days 6 and 7 there was massive development of leaf tissue as the leaves visibly enlarged, and at days 8–9 they began to unfold and separate as the internodes elongated. This was a time of intense biosynthetic activity with production of cuticular lipids and waxes and the constituents of the photosynthetic membranes, namely the chloroplast membrane acyl lipids, the chlorophylls and the carotenoids. This peak of mitochondrial β -oxidation was not observed when greening and leaf development were prevented by growing the plants in the dark (figure 1f). Peroxisomal β -oxidation was unaffected by etiolation (figure 1e).

The total β -oxidation in the radicles was low over the first 9 days of germination, rising gradually from 3 pmol palmitate oxidized per minute per radicle at day 3 to 15 pmol palmitate oxidized per minute per radicle at day 8 (figure 1g). This activity was largely due to peroxisomal β -oxidation, which accounted for 70–85% of the total activity (figure 1h). Mitochondrial β -oxidation activity remained at *ca.* 3.5 pmol palmitate oxidized per minute per radicle over these 9 days (figure 1i). The total radicle β -oxidation increased threefold at days 10–12 (figure 1g) and this peak of activity was due to an increase in mitochondrial β -oxidation, which accounted for 70% of the β -oxidation at this time (Figure 1i). Days 10–12 was the time-period when elongation of the primary root ceased and the secondary radicles proliferated and elongated, setting up a mature root system. This secondary root development was greatly retarded in dark-grown plants and once again etiolation resulted in reduced mitochondrial β -oxidation (figure 1i). Peroxisomal β -oxidation was unaffected by etiolation (figure 1e).

The total β -oxidation in the plumule decreased over the first 3 days of germination, from 5 pmol palmitate oxidized per minute per plumule at day 1 to 1.5 pmol palmitate oxidized per minute per plumule at day 3 and then began to rise again to an overall peak of 30 pmol palmitate oxidized per minute per plumule at day 13, before falling back to a rate of 9 pmol palmitate oxidized per minute per plumule at day 14 (figure 1j). The β -oxidation in the plumule was mainly accounted for by peroxisomal β -oxidation (figure 1k). Mitochondrial β -oxidation was virtually zero over the first 4 days (Figure 1l). Mitochondrial β -oxidation did rise to a peak of 12 pmol palmitate oxidized per minute per plumule at days 8 and 9, before falling and then rising to a second peak of 22 pmol palmitate oxidized per minute per plumule at day 13. Mitochondrial β -oxidation accounted for 75% of the β -oxidation rate at these two peaks, whereas peroxisomal β -oxidation accounted for 55–98% of the total β -oxidation rate at other times. The period of days 8 and 9 was a time when leaves were visibly enlarging and separating as the internodes elongated, and over days 11–13 the leaves were unfolding and expanding. Peroxisomal β -oxidation was unaffected when plants were grown in the dark (figure 1k), whilst the peak of mitochondrial β -oxidation at days 8 and

Figure 1. Palmitate oxidation rates in homogenates of pea seedlings and organs over a 14 day period following the commencement of imbibition. (a) Total, (b) peroxisomal and (c) mitochondrial palmitate oxidation rates of the whole seedling. (d) Total, (e) peroxisomal and (f) mitochondrial palmitate oxidation rates of the cotyledons. (g) Total, (h) peroxisomal and (i) mitochondrial palmitate oxidation rates of the radicles. (j) Total, (k) peroxisomal and (l) mitochondrial palmitate oxidation rates of the plumules. The results represent the mean $\pm$ standard deviation of six experiments. Where error bars are not visible they are occluded by the symbol. Open squares with solid lines represent light-grown plants and filled circles with dashed lines represent dark-grown (etiolated) plants.

9 was lost and the magnitude of the peak at day 13 was halved (figure 1l). Morphology was greatly altered in the etiolated plants. The leaves were small and yellow, and the internodes elongated.

Thus, the primary site of β -oxidation in developing pea seedlings is the cotyledon, the storage organ of leguminous seeds. The β -oxidation in the radicles and plumules remained low for the first 9 days following imbibition and

only increased in these organs after independent seedlings were established. Peroxisomal β -oxidation gave a low, steady baseline rate in all three organs and remained constant even when normal seedling development was disrupted by growth in the dark. It probably represents the site of essential β -oxidation activity, which is needed in all organs for removing harmful free fatty acids originating from both protein and membrane lipid turnover (Gerhardt 1992). In contrast, mitochondrial β -oxidation varied greatly, from zero to peaks of activity that were up to 17-fold higher than peroxisomal β -oxidation. These peaks coincide with the times of high biosynthetic activity associated with leaf and root development, and are not observed if normal development is disrupted by growth in the dark. Mitochondrial β -oxidation therefore appears to be switched on or off during seedling development.

It is possible that mitochondrial β -oxidation, which drives oxidative phosphorylation (Schulz 1991) and exports acetyl CoA via a carnitine acyltransferase system (Wood *et al.* 1992), may supply the acetyl CoA and/or ATP needed for the biosynthesis of membrane acyl lipids and photosynthetic pigments. Alternatively, the plant growth hormones, the cytokinins, are known to accelerate chloroplast development (Caers & Vendrig 1986) and leaf expansion (Letham 1971) and to retard primary root elongation (Su & Howell 1992) whilst promoting differentiation of vascular tissues in adventitious root growth (Aloni 1993), the very stages of development at which mitochondrial β -oxidation peaked. Cytokinins are synthesized from isopentenyl pyrophosphate, which in turn is synthesized from acetyl CoA in the isoprenoid pathway (McGaw 1995). Mitochondrial β -oxidation could supply acetyl CoA for this hormone synthesis.

It is clear from this work that mitochondrial β -oxidation is linked with the complex biochemical interactions associated with seedling development. It switches on, over and above the stable peroxisomal β -oxidation activity, at critical stages in development and plays a key role in the responses of lipid metabolism to developmental changes. Whilst much still remains unknown, what is clear is that future studies on the roles of both mitochondrial and peroxisomal β -oxidation will be crucial to an understanding of higher plant lipid metabolism.

The pea seeds were a kind gift from Batchelor Foods Ltd, Worksop, Nottinghamshire, UK. C. Masterson thanks the Royal Society for her university research fellowship.

REFERENCES

- Aloni, R. 1993 The role of cytokinin in organized differentiation of vascular tissues. *Aust. J. Plant Physiol.* **20**, 601–608.
- Bradford, M. M. 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.
- Caers, M. & Vendrig, J. C. 1986 Benzyladenine effects on the development of the photosynthetic apparatus in *Zea mays*—studies on photosynthetic activity, enzymes and (etio)chloroplast ultrastructure. *Physiol. Plant.* **66**, 685–691.
- Cooper, T. G. & Beevers, H. 1969 β -Oxidation in glyoxysomes from castor bean endosperm. *J. Biol. Chem.* **244**, 3514–3520.
- Douce, R. 1985 *Mitochondria in higher plants: structure, function and biogenesis*. New York: Academic Press.
- Gerhardt, B. 1992 Fatty acid degradation in plants. *Prog. Lipid Res.* **31**, 417–446.
- Kindl, H. 1993 Fatty acid degradation in plant peroxisomes: function and biosynthesis of the enzymes involved. *Biochimie* **75**, 225–230.
- Letham, D. S. 1971 Regulators of cell division in plant tissues. XII. A cytokinin bioassay using excised radish cotyledons. *Physiol. Plant.* **25**, 391–396.
- McGaw, B. A. 1995 Cytokinin biosynthesis and metabolism. In *Plant hormones. Physiology, biochemistry and molecular biology* (ed. P. J. Davies), pp. 98–117. Dordrecht, The Netherlands: Kluwer.
- Masterson, C. & Wood, C. 2000a Mitochondrial β -oxidation of fatty acids in higher plants. *Physiol. Plant.* **109**, 217–224.
- Masterson, C. & Wood, C. 2000b Contribution of mitochondria and peroxisomes to palmitate oxidation in pea tissues. *Biochem. Soc. Trans.* **28**, 759–762.
- Masterson, C., Blackburn, A. & Wood, C. 2000 Acyl CoA dehydrogenase activity in pea cotyledon tissue during germination and initial growth. *Biochem. Soc. Trans.* **28**, 762–764.
- Piot, C., Veerkamp, J. H., Bauchart, D. & Hocquette, J.-F. 1998 Contribution of mitochondria and peroxisomes to palmitate oxidation in rat and bovine tissues. *Comp. Biochem. Physiol.* **121**, 185–194.
- Schulz, H. 1991 β -Oxidation of fatty acids. *Biochim. Biophys. Acta* **1081**, 109–120.
- Su, W. P. & Howell, S. H. 1992 A single genetic locus, CKR1, defines *Arabidopsis* mutants in which root growth is resistant to low concentrations of cytokinin. *Plant Physiol.* **99**, 1569–1574.
- Wood, C., Masterson, C. & Thomas, D. R. 1992 The role of carnitine in plant cell metabolism. In *Plant organelles. Compartmentation of metabolism in photosynthetic tissue* (ed. A.K. Tobin), pp. 229–263. Cambridge University Press.