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The synthesis of short- and long-chain acylcarnitine by etio-chloroplasts of greening barley leaves

David R. Thomas*, M. Noh Hj Jalil[1], Azis Ariffin[2], Robert J. Cooke[3], Ian McLaren[4], Bob C.S. Yong and Clifford Wood
Department of Plant Biology, Newcastle University, Newcastle upon Tyne NE1 7RU, UK

Abstract. Etio-chloroplasts of barley, purified on sucrose density gradients were shown to possess carnitine long-chain acyltransferase (carnitine palmitoyltransferase, EC 2.3.1.21) activity and carnitine short-chain acyltransferase (carnitine acetyltransferase EC 2.3.1.7) activity. These enzymes may play a role in the transport of acyl groups as acylcarnitines through the membrane barrier of barley etio-chloroplasts and also 'or alternatively' may spare CoA by transferring short- and long-chain acyl groups from short- and long-chain acyl CoA to carnitine.

Key words: Acetylcarnitine – Carnitine acyltransferases – Chloroplast, etio- – Etio-chloroplast – *Hordeum* (acylcarnitine synthesis) – Palmitoylcarnitine.

Introduction

Thomas et al. (1982) used a linked-assay procedure to demonstrate the production of palmitoylcarnitine by purified etio-chloroplasts of barley. The assay required the presence in their preparations of both long-chain acyl CoA synthetase, (EC 6.2.1.3) and carnitine long-chain acyltransferase (carnitine palmitoyltransferase, EC 2.3.1.21). However, if long-chain acyl CoA synthetase was absent but carnitine long-chain acyltransferase was present in any fraction assayed then, necessarily, no palmitoylcarnitine would be formed in assay mixtures containing palmitate, CoASH, Mg^{2+}, ATP and carnitine which would satisfy reactions (i) and (ii)

$$\text{palmitate} + \text{CoASH} + \text{ATP} \rightarrow \text{palmitoyl CoA} + \text{AMP} + \text{PPi} \quad \text{(i)}$$

$$\text{palmitoyl CoA} + \text{carnitine} \rightleftharpoons \text{palmitoylcarnitine} + \text{CoASH} \quad \text{(ii)}$$

In the present work, palmitoyl CoA was supplied as substrate to fractions obtained after purification of washed etio-chloroplasts of barley on sucrose density gradients. This obviated the need for an active long-chain fatty acyl CoA synthetase (reaction i) hence providing a direct assay for carnitine long-chain acyltransferase (reaction ii). Also, the enzyme carnitine short-chain acyltransferase (carnitine acetyltransferase, EC 2.3.1.7) was assayed utilizing acetyl CoA as substrate in reaction (iii).

$$\text{acetyl CoA} + \text{carnitine} \rightleftharpoons \text{acetylcarnitine} + \text{CoASH} \quad \text{(iii)}$$

Material and methods

Growth of barley. Barley grain (*Hordeum vulgare* L. cv. Midas) was a gift from West Cumberland Farmers Ltd., Hexham, Northumberland. The grain was imbibed in running tap water overnight, planted in moist vermiculite and grown in total darkness at 25° C for 7 d. The barley was transferred to continuous white light (6.4 W m^{-2}) for 24 h. The light source was four, 40-W, "Universal White" fluorescent tubes (Atlas-GEC Ltd., Manchester, UK).

Chemicals. (−)-Carnitine, acetyl CoA, acetylcarnitine, palmitoyl CoA and palmitoyl-carnitine were purchased from Sigma (London) Chemical Co., Poole, Dorset, UK or the Boehringer Corporation (London) Ltd., Lewes, East Sussex, UK. Radio-

Present addresses:
[1] Cocoa and Coconut Branch, MARDI, Perak, Malaysia
[2] The Rubber Research Institute of Malaya, Kuala Lumpur, Malaysia
[3] Department of Biological Sciences, University of East Anglia, Norwich, UK
[4] Department of Biology, Sunderland Polytechnic, UK
* To whom correspondence should be addressed

chemicals were purchased from the Radiochemical Centre, Amersham, Bucks., UK. Bovine serum albumin, Fraction V powder, Sigma, was purified by the method of Thomas et al. (1982).

Preparation of etio-chloroplasts and assays of marker enzymes. Etio-chloroplasts were isolated from barley leaves and purified on sucrose density gradients as before (Thomas et al. 1982). Fractions off the sucrose density gradient were assayed for the enzymes fumarase (EC 4.2.1.2) (Hill and Bradshaw 1969), cytochrome c oxidase (EC 1.9.3.1) (Smith 1955) catalase (EC 1.11.1.6) (Aebi 1974), non-reversible $NADP^+$-linked glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9) (Kelly and Gibbs 1973) and chloroplast $NADP^+$-linked glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13) (Wolosiuk and Buchanan 1976). Chlorophyll was determined by the method of Arnon (1949). Sucrose concentrations were determined with an Abbé refractometer.

Assay of carnitine long-chain acyltransferase. The reaction mixture for the assay consisted of 250 mM sucrose, 1 mM $MgSO_4$, 0.3 mM palmitoyl CoA, 1 mM (−)-carnitine, 0.5% (w/v) bovine serum albumin, 0.1 ml H_2O containing 18.5 kBq (−, +)-methyl-[^{14}C]carnitine 2GBq mmol^{-1}), 20 mM 2-amino-2-(Chydroxymethyl)-1,3-propanediol (Tris)-HCl buffer pH 6.8. The reaction was started by the addition of 0.5 ml of the fraction under test. The final volume was 3 ml and the assay mixture was incubated for 45 min at 27° C in the dark. The reaction was ended by the addition of 1 ml 1 M HCl with, simultaneously, 1 mg palmitoylcarnitine as carrier. Palmitoylcarnitine in the acidified incubation mixtures was extracted with butan-1-ol by a method adopted from Bremer (1963) and used by Thomas et al. (1982). The volume of the final butan-1-ol extract was measured and 1 ml aliquots were dissolved in 10 ml toluene-based scintillant containing 5 g 2,5-diphenyloxazol and 0.3 g 1,4-bis-2′-(5′-phenyloxazolyl)benzene in 1 l toluene and counted in a Packard Tri-carb liquid scintillation spectrometer (Packard Instruments Ltd., Caversham, Berks. UK). The identity of palmitoylcarnitine formed in assays was checked by the procedures described previously (Thomas et al. 1982).

Assay of carnitine short-chain acyltransferase. The reaction mixture for the assay consisted of 250 mM sucrose, 1 mM $MgSO_4$, 10 µM acetyl CoA containing 4.63 mBq [^{14}C]acetyl CoA (1.85 GBq mmol^{-1}), 1 mM (−)-carnitine, 0.5% (w/v) bovine serum albumin, 20 mM Tris-HCl buffer pH 6.8. The reaction was started by adding 0.5 ml of the fraction under test. The final volume was 1 ml and the assay mixture was incubated for 45 min at 27° C in the dark. The reaction was ended by adding the 0.5 ml of the incubation mixture to a Dowex 2 × 8 − 400 Cl^--form column together with 1 mg acetylcarnitine as carrier. The resin was prepared in the Cl^--form (Cederblad and Linstedt 1972) and packed in 2-ml disposable syringes to form columns 1 cm diameter, 3.5 cm long. The mixture was allowed to elute very slowly through the column as previously described (Cederblad and Linstedt 1972; McNeil and Thomas 1975) and 0.5 ml aliquots of the eluate were added to 10 ml NE 260 scintillant (Nuclear Enterprises Ltd., Edinburgh, UK) and counted as before. Acetyl CoA was retained by the resin but acetylcarnitine passed through. This was verified in the present work by thin-layer chromatography (TLC) on 0.25-mm wet-thickness silica-gel (Kieselgel G; Merck, Darmstadt, FRG) plates in acidic solvent-methanol: acetone: HCl (90:10:4, by vol.), basic solvent-methanol: dioxan: NH_3 (specific gravity 0.88) (30:45:25, by vol.) (Eneroth and Lindstedt 1965). In eluates from the columns, only acetylcarnitine was detected. The Rf of acetylcarnitine in acidic solvent was 0.49 and in basic solvent 0.29. Spots on chromatograms were visualized by exposure to iodine vapour and radioactive spots by using a Radiochromatogram Spark Chamber No 450 B (Birchover Instruments Ltd., Herts., UK).

Isolation of crystalline acetylcarnitine from assay mixtures. When acetylcarnitine was required for crystallization to constant radioactivity, 0.5 mg (−)-carnitine and 50 mg acetylcarnitine were added as carriers on termination of the incubation which was carried out by layering 0.5 ml of the incubation mixture on top of a Dowex 2 × 8 − 400 column (Cl^--form). The eluate was evaporated to dryness and the residue dissolved in a small volume (3–4 ml) of boiling butan-1-ol. Crystals of acetylcarnitine were formed on cooling in ice. The crystals were harvested by centrifugation and dried in a desiccator under vacuum. A sample (2 mg) of dry crystals was dissolved in 0.5 ml H_2O in a counting vial, 10 ml NE 260 scintillant was added and the radioactivity determined as before. The acetylcarnitine was recrystallized from butan-1-ol to constant radioactivity. After the last crystallization, a sample of crystals was checked for purity and identity by TLC and mass spectrometry. Samples were analysed on an AEI MS902 spectrometer (A.E.I. Ltd., Manchester, UK).

Results and discussion

The distribution of marker enzymes and chlorophyll (Figs. 1 and 2) demonstrated that the etio-chloroplasts were collected between 19.5–27 ml, the mitochondria between 9–16.5 ml and the peroxisomes between 3–7.5 ml off the sucrose density gradients. The $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase, a soluble enzyme associated with intact chloroplasts (Wolosiuk and Buchanan 1976), was recovered almost exclusively in the etio-chloroplast fraction. There was little contamination of the etio-chloroplast fractions by marker enzyme except cytochrome-c oxidase and a little calatase activity which indicated that some inner mitochondrial membrane and peroxisome fragments were present. Carnitine long-chain acyltransferase was recovered principally in three peaks, the first coinciding with the cytosolic enzyme, non-reversible $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase (0–3 ml), the second with the mitochondrial markers, fumarase and cytochrome c oxidase (9–16.5 ml) and the third with the chloroplast markers, chlorophyll and glyceraldehyde-3-phosphate dehydrogenase (19.5–27 ml). Carnitine short-chain acyltransferase was recovered also principally as three peaks, the first coinciding with the peroxisome marker, catalase (3–7.5 ml), the second with the mitochondrial markers and the third with the etio-chloroplast markers.

A peak of carnitine long-chain acyltransferase activity was recovered in the first two fractions (0–3 ml) coinciding with the cytosolic marker enzyme. Thomas et al. (1982) did not find any activity for this enzyme in these fractions in their earlier

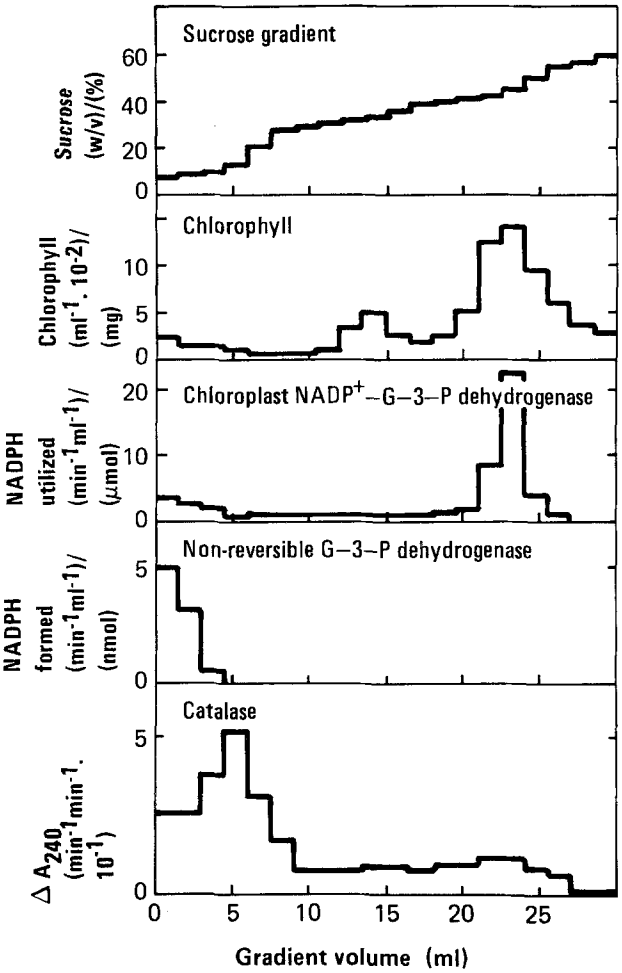

Fig. 1. Sucrose-density-gradient profiles of washed etio-chloroplasts of barley leaves. Distribution of sucrose, chlorophyll, chloroplast $NADP^+$-glyceraldehyde-3-phosphate (G-3-P) dehydrogenase, non-reversible G-3-P dehydrogenase and catalase

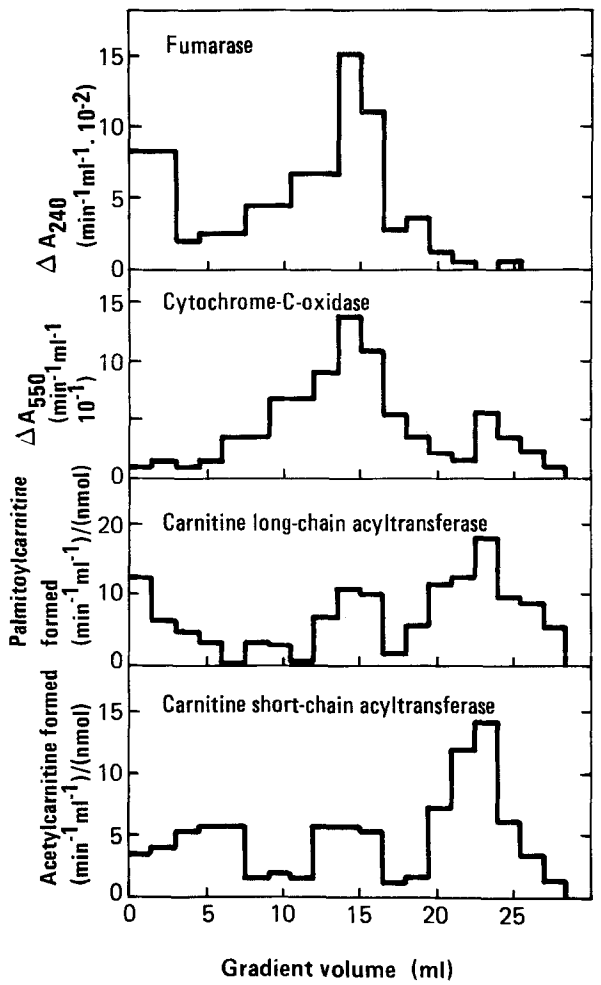

Fig. 2. Sucrose-density-gradient profiles of washed etio-chloroplasts of barley leaves. Distribution of fumarase, cytochrome-c oxidase, carnitine long-chain acyltransferase and carnitine short-chain acyltransferase

study of barley etio-chloroplasts but in their linked-assay system the presence of long-chain acyl CoA synthetase in the fractions was mandatory for the formation of palmitoylcarnitine. Hence it would seem that long-chain fatty acyl CoA synthetase was absent in these first fractions off the gradient and that carnitine long-chain acyltransferase can be detected in these first fractions only by the use of the direct assay employed in the present work. Whether or not this enzyme is located in the cytosol of the barley-leaf cell cannot be asserted on the current evidence, it may well have been derived from other organelles during the isolation procedure. The presence of carnitine short-chain acyltransferase in the first two fractions possibly may be accounted for by contamination with solubilized enzyme released from organelles during the experimental procedure. The peroxisome fractions (3–7.5 ml) were hardly contaminated with mitochondrial or chloroplast markers hence any carnitine acyltransferase activity is not likely to be derived from these organelles. It may be that carnitine short-chain acyltransferase is present in the barley-leaf peroxisome' although further work is required to confirm this suggestion.

Peaks of activity for both carnitine acyltransferase enzymes were recovered in the mitochondrial fractions. Previously (McNeil and Thomas 1976; Thomas and McNeil 1976; Thomas and Wood 1982) it had been shown that pea-cotyledon mitochondria required carnitine in order to oxidise palmitate and acetate at maximum rates and the presence of these enzymes in the mitochondria was inferred. Also, Thomas et al. (1982) using the linked assay concluded that carnitine long-chain acyltransferase was present in barley-leaf mitochondria, but a definite conclusion cannot be drawn from the data presented here since the mitochon-

Table 1. Recrystallization to constant specific activity of acetylcarnitine produced by purified etio-chloroplasts of greening barley leaves. The crystals were obtained by recrystallization from butan-1-ol (a) 18.5 kBq [^{14}C]acetyl CoA fed (b) 74 kBq [^{14}C]acetyl CoA fed

Crystallization step	Acetylcarnitine Bq (2 mg)$^{-1}$	
	(a)	(b)
1st	7.5	26.8
2nd	6.7	20.0
3rd	6.8	22.5
4th	6.7	22.8
5th	6.7	22.7

drial fraction is too contaminated by chloroplast markers.

Large peaks of activity for both carnitine acyltransferases were recovered in the etio-chloroplast fraction (Fig. 2). Contamination by any other cell organelle or fragment cannot account for the high activity of these enzymes in the etio-chloroplast fractions.

Proof of the identity of palmitoylcarnitine formed by barley etio-chloroplasts has been provided previously (Thomas et al. 1982). In the present work thin-layer chromatography (TLC) confirmed the identity of palmitoylcarnitine formed in the bulked cytosolic, mitochondrial and etio-chloroplast fractions (R_f 0.64 in acidic, R_f 0.61 in basic solvent). The radioactive palmitoylcarnitine co-chromatographed with authentic compound in both solvent systems. On alkaline hydrolysis, a single radioactive spot of carnitine was observed on TLC plates developed in either acidic (R_f 0.46) or basic (R_f 0.1) solvent.

The identity of acetylcarnitine in the bulked fractions of peroxisomes, mitochondria and etio-chloroplasts was revealed by TLC, providing one radioactive spot of acetylcarnitine in each solvent (R_f 0.49 in acidic solvent, R_f 0.28 in basic solvent). On hydrolysis, with KOH, of acetylcarnitine in the bulked fractions, a non-radioactive spot of carnitine was revealed with iodine vapour on TLC plates (R_f 0.46 in acidic solvent, R_f 0.1 in basic solvent).

Acetylcarnitine produced by the etio-chloroplast fractions after incubation was extracted with butan-1-ol and was recrystallized to constant activity after the third crystallization (Table 1). The identity of the purified crystals was supported by TLC as described previously and by mass spectrometry of the crystals obtained after the last crystallization. The mass spectra were identical to those published (Hvistendahl et al. 1970) and to those obtained with authentic compounds.

It is considered that the presence of carnitine short-chain acyltransferase and carnitine long-chain acyltransferase in barley etio-chloroplasts is confirmed. However, the precise location of these enzymes in the etio-chloroplast cannot be inferred from the data presented here. Thomas and his co-workers (Thomas and McNeil 1976; McNeil and Thomas 1976; Thomas and Wood 1982) suggested that carnitine-acyltransferase enzymes were located on the inner membrane of pea-cotyledon mitochondria. These enzymes would be involved in a transport system for acyl groups in the manner outlined by Thomas and Wood (1982) whereby the acylcarnitine formed would permeate the membrane more easily than the free aliphatic acid. They suggested that a 'cytosolic-side' extrinsic carnitine acyltransferase was linked to a 'matrix-side' extrinsic carnitine acyltransferase via an intrinsic translocator which carried the formed acetylcarnitine in one direction and free carnitine in the other. This transport system they considered to be freely reversible. If the location of these carnitine-acyltransferase enzymes in barley etio-chloroplasts is the membrane barrier, presumably the inner membrane, then it is possible to speculate that these enzymes may be involved in the transport of acyl groups across the membrane.

Carnitine may act as a saver of CoA and as a conserver of energy (McNeil and Thomas 1976). The standard free energy of the hydrolysis of the thioester group of acyl CoA is -30.7 KJ. Transfer of the acyl group from acyl CoA to carnitine in the reaction catalyzed by a carnitine acyltransferase involves a negligible change in the standard free energy of the system. CoASH is released and is available for other reactions but the original acyl-thioester-bond energy is conserved in the acyl-ester bond with carnitine. The acylcarnitine, it is suggested, freely permeates barley etio-chloroplast membranes in a manner similar to that suggested for acetylcarnitine (Thomas and Wood 1982) and palmitoyl-carnitine (McNeil and Thomas 1976) in pea-cotyledon mitochondria. The presence of the carnitine-acyltransferase enzymes on the membrane barriers of plant organelles may allow for a controlled flow of acyl groups between the various organelles of the plant cell. Liedvogel and Stumpf (1982) and Murphy and Walker (1982) suggested that a chloroplast source of acetyl groups may be derived from mitochondrial-matrix acetyl CoA and they envisage an hydrolysis of the acetyl CoA (energy loss 30.7 KJ) and the free acetate then moves to the chloroplast where it reforms

acetyl CoA (energy loss 30.7 KJ). Instead it is proposed that acetyl CoA in the mitochondrial matrix is converted to acetylcarnitine which then is transported to the chloroplast-matrix sites without excessive loss of energy. Such traffic is postulated to be freely reversible according to the model presented for pea-cotyledon mitochondria (Thomas and Wood 1982). Currently, the factors which control the direction of the flow of acylcarnitine and the precise location of the carnitine acyltransferases are being studied.

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