Pergamon

Phytochemistry, Vol. 39, No. 1, pp. 39–43, 1995

0031-9422/95 $9.50 + 0.00

0031-9422(94)000922-8

PURIFICATION AND CHARACTERIZATION OF CARNITINE ACYLTRANSFERASE FROM HIGHER PLANT MITOCHONDRIA

HEIDRUN SCHWABEDISSEN-GERBLING* and BERNT GERHARDT†

Unité mixte Rhône-Poulenc Agrochimie, 14–20 rue Pierre Baizet, 69263 Lyon, France; †Institut für Botanik der Universität Münster, Schloßgarten 3, 48149 Münster, Germany

(*Received in revised form* 31 *October* 1994)

Key Words Index—*Vigna radiata*; Fabaceae; mung-bean; purification; carnitine acyltransferase; mitochondria.

Abstract—Carnitine acyltransferase was purified to homogeneity from mung-bean (*Vigna radiata* L.) hypocotyl mitochondria. The native enzyme has an apparent M_r of 45 000 as measured by gel filtration. SDS–PAGE revealed the same indicating a monomeric structure. The enzyme is active with short- and long-chain acyl-CoAs. The activity ratio determined for the substrate acetyl-CoA and palmitoyl-CoA remained the same throughout purification. The ability of the enzyme to use acetyl-CoA and palmitoyl-CoA as substrates is unique amongst the carnitine acyltransferases characterized to date. Apparent K_m values for the enzyme substrates acetyl-CoA, palmitoyl-CoA, and L-carnitine were: 8.5, 2.5 and 5 μM, respectively.

INTRODUCTION

Carnitine acyltransferases catalyse the reversible transfer of a fatty acyl-group from acyl-CoA to L-carnitine in order to faciliate the transport of acyl-moieties across membranes. Depending on the acyl-CoA favoured to transacylate onto carnitine, the enzymes were differentiated into carnitine acetyltransferases (CAT, EC 2.3.1.7) which are active with short-chain acyl-CoAs, carnitine octanoyltransferases (COT, EC 2.3.1.137) which are active with medium-chain acyl-CoAs, and carnitine palmitoyltransferases (CPT, EC 2.3.1.21) acting on long-chain acyl-CoAs. Purified CAT, COT and CPT from rat liver are immunologically distinct [1]. In this paper, the term 'carnitine acyltransferase' is used when discussing the enzyme in general or describing the plant enzyme. The more specific terms of CAT, COT and CPT are used when referring to precisely described enzymes from mammalian tissues. In mammalian cells, carnitine acyltransferases are located on both sides of the inner mitochondrial membrane, in the peroxisomal membrane, and in microsomes from some tissues. Current knowledge of plant carnitine acyltransferases is rather meagre. The presence of carnitine acyltransferases in plant cells has been reported for mitochondria and chloroplasts [2–5], but the chloroplastic occurrence has been questioned [6].

Plant mitochondria exhibit carnitine acyltransferase activity which could be demonstrated using both short- and long-chain acyl-CoAs [4, 5, 7]. As carnitine acyltransferase activity was demonstrated with acetyl-CoA and palmitoyl-CoA as substrates, the presence of both carnitine acetyltransferase and carnitine long-chain acyltransferase in pea mitochondria has been claimed [8, 9]. Inhibition studies performed on the mitochondrial carnitine acyltransferase activities from mung-bean hypocotyl gave hints for the presence of only one single enzyme, active with short-, medium- and long-chain acyl-CoAs as substrates [5, 10]. The present investigation was undertaken in an attempt to purify the mitochondrial inner membrane-bound carnitine acyltransferase(s) whose function in the plant mitochondria is/are not yet established.

*Author to whom correspondence should be addressed (Fax 33-72-29-22-97).

RESULTS AND DISCUSSION

Carnitine acyltransferase was purified from mitochondria isolated from mung-bean hypocotyls (Table 1). Attempts were not undertaken to purify the enzyme from crude extract because of the instability of the enzyme and possible contamination by other carnitine acyltransferases present in the cell. The mitochondrial fractions were not contaminated by plastids [11], and peroxisomes which occasionally contaminate mitochondrial preparations can be ruled out as the origin of the enzyme, because they lack carnitine acyltransferase activity [1, 5, 12].

Solubilization of the membrane-bound carnitine acyltransferase was the crucial step in the purification procedure. Following incubation of mitochondria in the

Table 1. Purification of carintine acyltransferase from mung-bean hypocotyl mitochondria

Step	Total protein (mg)	Total activity with ACoA (nkat)	Total activity with PCoA (nkat)	Specific activity with ACoA (nkat mg^{-1} protein)	Specific activity with PCoA (nkat mg^{-1} protein)	Ratio	Recovery (%)	Purification (-fold)
Broken mitochondria	60	4200	5400	70	90	1.28	100	1
Green A-agarose chromatography	12	4032	5060	336	420	1.26	95	4.7
Gel filtration	0.12	870	1150	7248	9550	1.31	21	105

presence of $MgCl_2$ and Triton X-100, centrifugation of the broken mitochondria did not result in a solubilized carnitine acyltransferase but the activity remained in the pellet, i.e. the membrane fraction. This fraction had to be loaded immediately onto a chromatography column in order to separate the carnitine acyltransferse from the membrane components without losing its activity. The chromatographic separation could be achieved using gel matrices as different as hydroxyapatite or DEAE (not shown), but the best results were obtained with Green A-agarose. Over 90% of the initial mitochondrial activity could be recovered in the Green A-eluate (Fig. 1). After concentration of this enriched fraction the enzyme was purified further by gel filtration. The purification procedure resulted in an overall 100-fold purification. The yield was about 20% (Table 1).

The purification procedure described provides carnitine acyltransferase in good yield and high purity in a short working time. Unfortunately, the purified enzymes is not very stable and can only be stored in the presence of glycerolipids (Table 2). Interestingly, lost activity could be restored through the presence of palmitoylcholine or similar compounds.

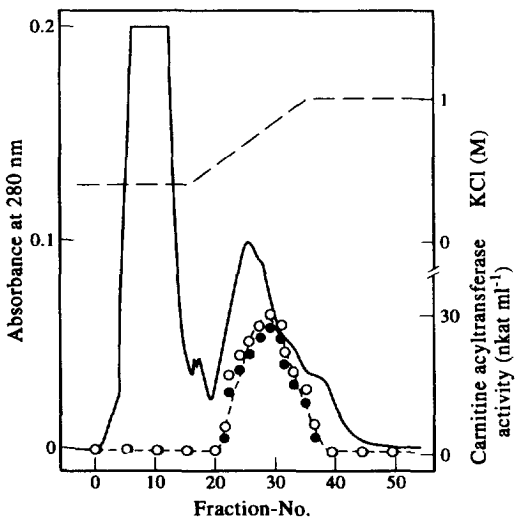

Fig. 1. Elution profiles of the Green A-agarose chromatography of broken mitochondria from mung-bean hypocotyls. For details see Experimental. Carnitine acyltransferase activities with O—O palmitoyl-CoA, and ●—● acetyl-CoA as substrate; —— protein elution patterns (280 nm); ---- concentration of KCl.

On the basis of SDS–PAGE, the mitochondrial carnitine acyltransferase from mung-bean hypocotyls is a monomer with a M_r of 45 000 (Fig. 2). The apparent M_r of the native enzyme was estimated by gel filtration chromatography on a Superdex 200 column (Pharmacia) and was also found to be 45 000. Similar characteristics were reported for carnitine acetyltransferases (CATs) throughout the mitochondria from mammalian tissues which are monomers with comparable molar masses [1, 13]. They differ from characteristics of mammalian CPTs which have very high M_rs and are heteromeric enzymes composed of several subunits [1, 14, 15]. The differences might be linked to different hydrophobic properties of carnitine acyltransferases.

Isoelectric focusing of the the purified carnitine acyltransferase revealed an isoelectric point of pH 6.0 (data not shown). The isoelectric point corresponds to that determined for commercial CAT (pH 6.0) and to values reported for CAT and COT purified from mouse liver peroxisomes (pH 6.8 and pH 5.2, respectively [16]).

The K_m values of the carnitine acyltransferase purified from mung-bean hypocotyls for L-carnitine, ACoA and PCoA differ from those reported for mammalian carnitine acyltransferases [1]. The carnitine acyltransferase from mung-bean mitochondria also exhibits a broad substrate specificity including fatty acids from C_2 to C_{18} chain length (Table 3). This broad substrate specificity is completely different from the rather limited substrate specificity of mammalian carnitine acyltransferases. At this point it was interesting to investigate whether the carnitine acyltransferase from mung-bean mitochondria possesses different reaction sites for substrates of different chain length. Therefore, the so called 'competition plot' was employed [17]. The total rate of reactions was measured for mixtures of substrates (ACoA and PCoA) at concentrations $(1 - p)$ $ACoA_0$ and $pPCoA_0$ where the reference concentrations $ACoA_0$ and $PCoA_0$ were chosen so that they gave equal rates in experiments with only one substrate present. The total rate was plotted

Table 2. Effect of lipidic compounds and polyalcohols on the stability ofpurified carnitine acyltransferase

Concentration (mM)	Compound	(Source)	Activity (%)
—	without		0
0.35	palmitoylcholine	(synthetic)	190
0.35	phospatidylcholine	(bovine brain)	180
0.35	phosptidylinositol	(bovine liver)	135
0.35	phosphatidylethanolamine	(bovine brain)	180
0.42	phosphatidylglycerol	(egg yolk)	120
10%	glycerol		10
10%	polyethylene glycol 6000		185

50 μg carnitine acyltransferase were stored for 1 month at $-20°$ without and in the presence of the listed compounds. 100% represents an activity of 5.4 μkat mg^{-1} protein with PCoA as substrate, determined before storage.

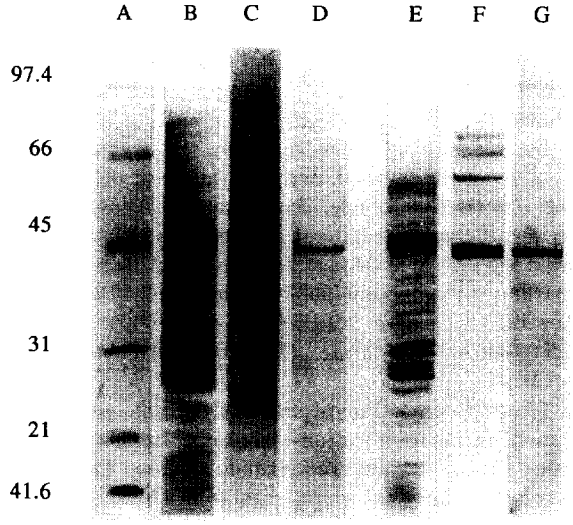

Fig. 2. SDS–PAGE documentation of the purification of mung-bean mitochondrial carnitine acyltransferase. Polypeptides were stained with Coomassie Brillant Blue R-250 (lanes A–D) or with the silver staining procedure (lanes E–G). Lane A, M_r markers, 8 μg each; lane B, broken mitochondria, 500 μg protein; lane C, Green A-agarose eluate 50 μg protein; lane D, purified carnitine acyltransferase, 15 μg protein; lane E, broken mitochondria, 150 μg protein; lane F, Green A-agarose eluat, 70 μg protein; lane G, purified carnitine acyltransferase, 15 μg protein. Molecular masses are given in kD.

Table 3. Substrate specificity of carnitine acyltransferase purified from mung-bean mitochondria

Substrate	Activity
Acetyl-CoA	85
Octanoyl-CoA	100
Palmitoyl-CoA	100
Oleoyl-CoA	105
Linoleoyl-CoA	108
Linolenoyl – CoA	130

Acyl-CoA substrates were present ata final concentration of 10 μM. Activities were determined by the release of CoASH from acyl-CoA. 100% activity corresponds to 9.0 μkal mg^{-1} protein.

against *p*. The velocity rate with acyl-CoA mixtures was constant, independent of the proportions of the two substrates. The total velocity did not distinguish between the two different reactions. Therefore, the two substrates competed and were metabolized for/at the same active site of the carnitine acyltransferase without antagonistic effects (Fig. 3).
without antagonistic effects (Fig. 3).

The activity of the mitochondrial carnitine acyltransferase from mung-bean hypocotyls was not modulated by DTNB. Preincubation of reaction mixtures with DTNB (6 mM) did not lead to an alteration of the enzyme activity within 30 min at room temperature. DTNB was occasionally reported to inhibit or to stimulate CAT or COT from mammalian tissues [16]. The presence of malonyl-CoA (up to 50 μM) in the reaction mixture did not lead to an inhibition of the enzyme activity. This is in accordance with results reported for COT and CAT from mouse liver peroxisomes [16, 18]. However, the result is in contrast with the observations that malonyl-CoA influences mammalian mitochondrial COT and CPT activities; this effect is directly related to the regulation of β-oxidation of long- and medium-chain fatty acids in mammalian mitochondria [16, 18].

The mitochondrial carnitine acyltransferase from mung-bean hypocotyls did not exhibit serine palmitoyltransferase activity when carnitine was replaced by serine (2 mM) in the presence of pyridoxal 5′-phosphate (50 μM) in the assay mixture. Serine palmitoyltransferase was reported as a pyridoxal 5′-phosphate-depending enzyme present in plant chloroplasts [19].

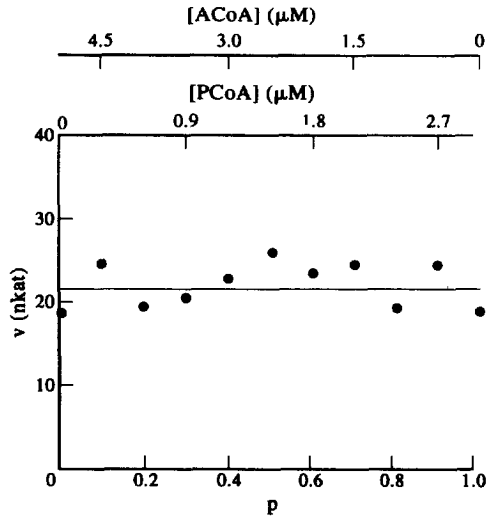

Fig. 3. Competition plot showing transacylation of acyl-CoAs by carnitine acyltransferase. Transacylation of acetyl-CoA and palmitoyl CoA at concentrations $(1-p)\times 5\ \mu M$ and $p\times 3\ \mu M$, respectively, catalysed by mitochondrial carnitine acyltransferase. p varied from 0 to 1. Transacylation was measured by the release of CoASH from acyl-CoA, using the coupling reaction with DTNB. Assay conditions as described in the Experimental. The reactions were started by adding L-carnitine. The experiment was repeated five times. The velocity rate with acyl-CoA mixtures was constant, independent of the proportions of the two substrates. The total velocity did not distinguish between the two different reactions. Therefore, the two substrates competed for the same active site.

According to the K_m values obtained, the mitochondrial carnitine acyltransferase from mung-bean hypocotyls should be classified as a CPT. However, other properties of the enzyme differ considerably from those of the CPTs characterized from mammalian tissues. Many biochemical properties (small size, monomeric structure) of the enzyme resemble rather those of a CAT in spite of the good activity with palmitoyl-CoA. Different metabolic functions of the organelles in mammalian and plant tissues might be reflected in the properties of the carnitine acyltransferases.

The presence of other carnitine acyltransferases in plant mitochondria specifically active with short- or long-chain acyl-CoAs, as mentioned in the introduction, cannot be excluded. On the other hand, results indicating the presence of more than one mitochondrial carnitine acyltransferase were obtained using non-purified preparations which do not exclude the possibility that the activities were due to one protein, the now purified mitochondrial carnitine acyltransferase.

EXPERIMENTAL

Plant material and prepn of mitochondria. Seedlings of mung-bean (*Vigna radiata* L.) were grown in the dark and mitochondria were isolated from hypocotyls of 2- to 3-day-old seedlings as described previously [11].

Purification of the membrane-bound carnitine acyltransferase from mung-bean mitochondria. Mitochondria (50 ml) isolated on sucrose density gradients were diluted with 50 ml H_2O and centrifuged for 15 min at 41 000 *g*. The pellet was resuspended (about 7 mg protein ml^{-1}) in 6 ml of 10 mM K-Pi buffer (pH 7.6), containing 50 mM $MgCl_2$ and 0.02% Triton X-100. After 1 hr incubation at 4° the mixt. (broken mitochondria) was loaded onto a Reactive Green A-(cross-linked 5% agarose with covalently coupled dye)-column (Amicon, U.S.A.) previously equilibrated with 25 mM K-Pi buffer, pH 7.6. Unbound proteins were removed by washing with 50 ml of the same buffer. Carnitine acyltransferase was eluted from the column by an 80 ml gradient consisting of 0 to 1 M KCl in 25 mM K-Pi buffer, pH 7.6. The flow rate was 1 ml min^{-1}. Active frs were pooled and overnight concd in the presence of palmitoylcholine iodide (or polyethylene glycol) (12 μM at the beginning of the concn), using Macrosep-10 tubes (Filtron). The final protein concn amounted to *ca* 6 mg ml^{-1}. The concd Green A-eluate was loaded onto a Superdex 200 column equilibrated with 25 mM K-Pi buffer (pH 7.6). The flow rate was 1.5 ml min^{-1}. After chromatography active frs were pooled and directly assayed.

Determination of native M_r *of carnitine acyltransferase.* The M_r of the purified, native carnitine acyltransferase was estimated on a Superdex 200 column, using the same chromatography conditions as above. The M_r was calcd from the plot of ln M_r against elution vol., using the M_r markers thyroglobulin (668 700), catalase (240 000), alcohol dehydrogenase 150 000), BSA (66 000), carnitine acetyl-CoA transferase (58 000), and cytochrome *c*(12 400).

Electrophoresis. Polypeptides of the different frs obtained during purification were sepd by SDS–PAGE using a 7.5 to 15% (w/v) acrylamide gradient. The experimental details of gel prepn, sample solubilization, electrophoresis and gel staining were as reported elsewhere [20]. Silver staining of gels was performed as described in ref. [21].

Isoelectric focusing. Concd carnitine acyltransferase was focused for 3 hr in the range of pH 2 to pH 11 according to [22]. After focusing, the gel was cut into thin slices which were incubated with slight agitation for 12 hr in assay buffer at 4°. Activity was determinated in the elutions.

Assay for carnitine acyltransferase. Carnitine acyltransferase activity was measured in the direction of formation of acyl carnitine from acyl-CoA. The appearance of the free SH-group of the released CoASH was followed spectrophotometrically by its reaction with 5,5′-dithio-bis-(2-nitrobenzoate) (DTNB, Ellman's reagent). The reaction mixt. (1 ml total vol.) consisted of 400 mM Tris–HCl (pH 7.6), 4 mM KH_2PO_4, 1 mM $MgCl_2$, 1 mM EDTA, 6 mM DTNB, 10 μM acyl-CoA, 1 mM L-carnitine, and different amounts of protein. Acyl-CoA or L-carnitine were omitted for the blanks. The increase of A at 412 nm ($\varepsilon_{412} = 13.6\ cm^2\ \mu mol^{-1}$) was followed.

With purified prepns the cleavage or formation of the thioester bond of acyl-CoA catalysed by carnitine acyl-

transferase, was assayed by recording the decrease of increase in A at 232 nm ($\varepsilon_{232} = 5.0\,cm^2\,mol^{-1}$). DTNB was omitted from these reaction mixts, and L-carnitine and acyl-CoA were also omitted when palmitoyl-carnitine (10 μM) and CoASH (1 mM) were used as substrates.

Protein determination. Protein was measured by the method of [23] using the Bio-Rad protein assay reagnet with γ-globulin as standard.

Acknowledgements—The authors gratefully acknowledge the contributions of Dr Claude Alban during the practical work and the preparation of the manuscript. The study was supported by the Deutsche Forschungsgemeinschaft.

REFERENCES

1. Bieber, L. L. (1988) *Ann. Rev. Biochem.* **57**, 261.
2. Thomas, D. R., Jalil, M. N. R., Ariffin, A., Cooke, R. J., McLaren, I., Yong, B. C. S. and Wood, C. (1983) *Planta* **158**, 259.
3. McLaren, I., Wood, C., Jalil, M. N. H., Yong, B. C. S. and Thomas, D. R. (1985) *Planta* **163**, 197.
4. Thomas, D. R. and Wood, C. (1986) *Planta* **168**, 261.
5. Gerbling, H. and Gerhardt, B. (1988) *Planta* **174**, 90
6. Roughan, G., Post-Beittenmiller, D., Ohlrogge, J. and Browse, J. (1993) *Plant Physiol.* **101**, 1157.
7. Burgess, N. and Thomas, D. R. (1986) *Planta* **167**, 58.
8. Wood, C., Jalil, M. N. R., Ariffin, A., Yong, B. C. S. and Thomas, D. R., (1983) *Planta* **158**, 175.
9. Wood, C., Jalil, M. N. R., McLaren, I., Yong, B. C. S., Ariffin, A., McNeil, P. H., Burgess, N. and Thomas, D. R. (1984) *Planta* **161**, 255.
10. Gerbling, H., Gandour, R. D., Moore, T. S. and Gerhardt, B. (1990) in *Plant Lipid Biochemistry, Structure, Function and Utilization* (Quinn, P. J. and Harwood, J. L., eds), p. 181. Poland Press, London.
11. Gerbling, H. and Gerhardt, B. (1987) *Plant Physiol.* **67**, 341.
12. Miernyk, J. A. and Trelease, R. N. (1981) *Plant Physiol.* **16**, 307.
13. Colucci, W. J. and Gandour, R. D. (1987) *Bioorg. Chem* **16**, 307.
14. Bergström, J. D. and Reitz, R. C. (1980) *Arch. Biochem. Biophys.* **204**, 71.
15. Clarke, R. H. and Bieber, L. L. (1981) *J. Biol. Chem.* **256**, 9861.
16. Farrel, S. O., Fiol, C. J., Reddy, J. K. and Bieber, L. L. (1984) *J. Biol. Chem.* **259**, 13089.
17. Chevillard, C., Cardenase, M. L. and Cornish-Bowden, A. (1993) *Biochem. J.* **289**, 599.
18. Moir, A. M. B., and Zammit, V. A. (1993) *Biochem. J.* **291**, 214.
19. Lynch, D. V. and Fairfield, S. R. (1993) *Plant Physiol* **103**, 1421.
20. Chua, N. H. (1980) *Methods Enzymol.* **69**, 434.
21. Wray, W., Boulikas, T., Wray, V. and Hancock, R. (1981) *Anal. Biochem.* **118**, 197.
22. Robertson, E. F., Dannelly, H. K., Molloy, P. J. and Reeves, H. C. (1987) *Anal. Biochem.* **167**, 290.
23. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248.