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DETERMINATION OF OVERT CARNITINE PALMITOYLTRANSFERASE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the determination of carnitine palmitoyltransferase I (CPT I; EC 2.3.1.19) in isolated rat liver mitochondria by reversed-phase high-performance liquid chromatography is described. Enzyme activity is assayed by direct determination of coenzyme A (CoA) released from palmitoyl-CoA within 60 min by a linear gradient system. CPT I in rat liver mitochondria can be assayed from only 30 μg of mitochondrial protein per millilitre of assay mixture. The changes in the kinetic parameters of CPT I, including K_i for malonyl-CoA, resulting from the fasting–feeding cycle are also discussed.

INTRODUCTION

Carnitine palmitoyltransferase (CPT; EC 2.3.1.19) catalyses the transfer of long-chain acyl coenzyme A (acyl-CoA) into mitochondria for β-oxidation [1]. CPT is thought to exist exclusively in the mitochondria [2] and is found on both sides of the mitochondrial inner membrane in its overt (CPT I) and latent (CPT II) forms, respectively [3,4]. Some studies have indicated that malonyl-CoA, which is the first intermediate in lipogenesis, is a potent inhibitor of CPT I [5–14]. Both the CPT I activity and the sensitivity to malonyl-CoA inhibition depend on the nutritional [6–10] and hormonal states [7,11–14] of the experimental animal, and it is probable that the interaction of these two factors modulates the partitioning of long-chain acyl-CoAs between oxidation

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and esterification [15]. Thus, to understand the regulatory mechanism of the flux of long-chain acyl-CoA, a method is required for the measurement of CPT I activity and the sensitivity of CPT I to malonyl-CoA inhibition.

The assay generally employed currently for measuring CPT I in isolated mitochondria [6] and permeabilized hepatocytes [12,14] involves the incorporation of a ^{14}C label into palmitoylcarnitine from [^{14}C]carnitine and palmitoyl-CoA. The radioisotopic method is sensitive and reproducible, but it requires expensive radioisotopes and the institution of radioisotope management.

We have developed a new method for the determination of CPT I activity in mitochondrial preparations by high-performance liquid chromatography (HPLC), which does not involve the use of radioisotopes. CPT I activity was assayed by the direct determination of coenzyme A (CoASH) released from palmitoyl-CoA and carnitine using reversed-phase HPLC. The analysis of CPT I using HPLC is characterized not only by its reliability, but also by its sensitivity, which is comparable with that of the radioisotopic assay. Application of this method for the examination of the kinetic parameters of CPT I in rat liver is also demonstrated.

EXPERIMENTAL

Chemicals

CoASH and malonyl-CoA were purchased from Sigma (St. Louis, MO, U.S.A.). Dithiothreitol, β-thiodiglycol and all other reagents were purchased from Wako (Osaka, Japan). L-Carnitine was kindly donated by Earth Chemical Company.

CoASH and malonyl-CoA were prepared as a 1 m*M* stock solution in 10 m*M* NaH_2PO_4 containing 5 m*M* dithiothreitol, and stored at $-70°C$ or below.

Apparatus

A Shimadzu Model LC-4A HPLC system equipped with a Model SIL-2AS automatic loop injector, a Model SPD-2AS UV spectromonitor, a Model CTO-2AS column oven and a Model CR-2AX reporting integrator (Shimadzu, Kyoto, Japan) was used. Chromatographic separations were carried out with a 250 mm × 4.0 mm I.D. LiChrosorb RP-18 (5 μm particle size) analytical column (Wako) and a 45.0 mm × 4.0 mm I.D. Zorbax ODS (45 μm particle size) guard column (Wako). The column effluent was monitored at 254 nm. The sensitivity of the UV detector was 0.04 a.u.f.s.

The spectrophotometer used was a Shimadzu Model UV 260 (Shimadzu, Kyoto, Japan).

Isolation of mitochondria

Wistar strain male rats weighing 180–220 g were used in two groups. One group ('the fasted group') was starved for 48 h before use and the other ('the

fed group') was fed on a standard pellet diet. The animals were decapitated, and immediately after this a 30% (w/v) liver homogenate was prepared in ice-cold 0.25 *M* sucrose containing 2 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) at pH 7.4, and 1 m*M* ethylene glycol tetraacetate (EGTA) with a Potter–Elvehjem PTFE glass homogenizer. Liver mitochondrial fractions were obtained by differential centrifugation according to the method of Johnson and Lardy [16]. The final mitochondrial pellet was washed twice with homogenization buffer in order to remove unwanted compounds, in particular malonyl-CoA, which interfere with the accuracy of the assay, from the mitochondrial preparations, and then resuspension was carried out to give a protein concentration of 40 mg/ml in 0.3 *M* sucrose, 1 m*M* EGTA and 2 m*M* HEPES, at pH 7.2. The resulting suspension was used for CPT I assays within 30 min. Each preparation of mitochondria was tested for intactness by the respiratory control ratio method. The respiratory control ratios were 4.5 or greater with 10 m*M* glutamate and 0.5 m*M* malate as substrates. Mitochondrial protein was measured with a Bio-Rad protein assay kit (Bio-Rad Labs., U.S.A.) with bovine plasma γ-globulin as a standard [17].

CPT I assay

The assay mixture was prepared according to Bremer [6] with small modifications: dithiothreitol was modified to 5 m*M* and the pH was lowered to 7.0. Briefly, the incubation mixture contained, in a final volume of 1.0 ml, 75 m*M* potassium chloride, 50 m*M* mannitol, 25 m*M* HEPES (pH 7.0), 0.2 m*M* EGTA, 2 m*M* potassium cyanide, 5 m*M* dithiothreitol, 1.75 mg of essentially fatty acid-free bovine serum albumin, palmitoyl-CoA (30–120 μM), 0.5 m*M* L-carnitine, malonyl-CoA (0–150 μ*M*) and mitochondrial suspension. The reference solution was made with identical ingredients, except that distilled water was substituted for L-carnitine. These assay mixtures, before the addition of the mitochondrial suspension, were preincubated for 2 min at 25°C, and the reaction was then initiated by adding 100 μl of the mitochondrial suspension (0.1–0.3 mg of protein) to both mixtures. After 5 min of incubation at 25°C, the reactions were terminated by the addition of 50 μl of 60% (v/v) perchloric acid, and the mixtures were centrifuged for 15 min at 4000 *g* at 4°C. By this procedure, the remaining palmitoyl-CoA (acid-insoluble acyl-CoA) was precipitated. The resultant supernatant, 800 μl, was adjusted to pH 2–3 by the slow addition of 2 *M* potassium phosphate with continuous vortex-mixing to avoid local alkalinity in the solution. The neutralized mixture was cooled in ice for 30 min, then centrifuged for 10 min at 18 000 *g* and 4°C to remove the precipitated potassium perchlorate. The supernatant containing released CoASH was filtered through a Millipore HV4L column filter (0.45 μm). A portion of this filtrate (generally 50 μl) was injected directly into the chromatograph.

The sensitivity of CPT I to inhibition by malonyl-CoA was assessed by em-

ploying the graphical method of Dixon [18] and by using 50 μM and 80 μM palmitoyl-CoA, 0.5 mM L-carnitine and malonyl-CoA at concentrations of 0, 0.25, 0.4, 0.5, 0.8, 1, 2 and 2.5 μM. The lines of the Dixon plot were linear below malonyl-CoA concentrations of 2.5 μM for starved animals and 1.0 μM for fed animals.

Chromatography

Chromatography was performed at 40°C using a linear gradient, at a flow-rate of 0.5 ml/min, as described elsewhere [19], with small modifications: NaH_2PO_4 was used in the mobile phase instead of KH_2PO_4, and the gradient condition was also modified. Solvent A contained 220 mM NaH_2PO_4 and 0.05% (v/v) β-thiodiglycol and solvent B was prepared from 125 mM NaH_2PO_4, 43% (v/v) methanol, 0.9% (v/v) chloroform, and 0.05% (v/v) β-thiodiglycol. β-Thiodiglycol was added to these solvents to prevent the oxidation of CoASH during analysis. The solvent was degassed by vacuum and filtered through a 0.45-μm filter. The composition of the mobile phase was 88% solvent A plus 12% solvent B at time 0, 85% solvent A plus 15% solvent B at 8 min, 35% solvent A plus 65% solvent B at 28.5 min and 100% solvent B at 29–31 min. The original compositions were reestablished by a reverse gradient to 88% solvent A plus 12% solvent B within the next minute. This composition was maintained for 28 min from 32 to 60 min, at which time the column was ready for injection of the next sample.

The column maintained its satisfactory resolution capacity for 400–500 injections. Complete regeneration of the column was achieved by washing it each day with 100% methanol until a flat baseline was obtained.

RESULTS AND DISCUSSION

Fig. 1 shows a representative elution profile of a mixture of CoASH, malonyl-CoA and dithiothreitol. Good separation of the compounds was obtained with the reversed-phase column within 60 min. For injected CoASH in the range of 20 pmol (signal-to-noise ratio higher than 3:1) to 2 nmol, the detector response was linear ($r^2 \geqq 0.98$).

Elution profiles for the isolated rat liver mitochondrial CPT I, using the standard assay procedure, are presented in Fig. 2. Fig. 2a shows the profile of the CoASH released from palmitoyl-CoA in the presence of L-carnitine and Fig. 2b shows that of the CoASH released from palmitoyl-CoA in the absence of L-carnitine. In the latter instance, the CoASH resulted mainly from the presence of palmitoyl-CoA hydrolase, which produces CoASH from palmitoyl-CoA and partially from non-enzymic palmitoyl-CoA hydrolysis and the contamination of CoASH in isolated mitochondria. The CoASH release brought about by carnitine acyltransferase was obtained by subtracting the CoASH from which Fig. 2b was obtained from the CoASH used to obtain Fig. 2a.

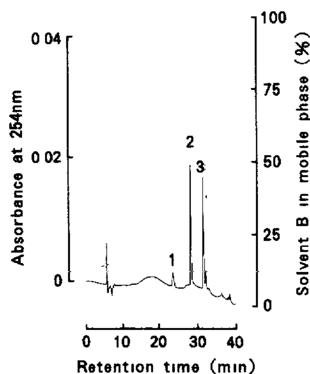

Fig. 1. HPLC profile of authentic coenzyme standards: 50-μl aliquots containing 200 pmol of malonyl-CoA and CoASH and 100 pmol of dithiothreitol were injected. Peaks: 1 = dithiothreitol; 2 = malonyl-CoA; 3 = CoASH. Column, LiChrosorb RP-18 (5 μm, 250 mm $\times$ 4.0 mm I.D.); oven temperature, 40°C; flow-rate, 0.5 ml/min; chart speed, 0.2 cm/min.

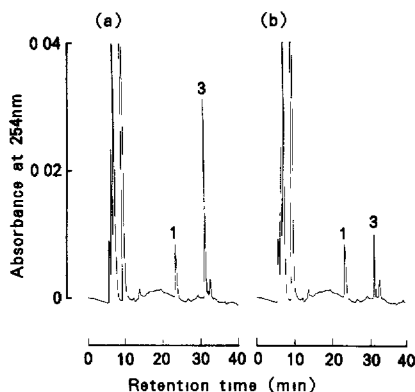

Fig. 2. HPLC profiles of representative CPT I assays using liver mitochondria of rats starved for 48 h. The assay mixtures containing L-carnitine (a) and without L-carnitine (b) were incubated with freshly prepared mitochondria (200 μg of protein). Assays were performed, as described in Experimental, using 80 μM palmitoyl-CoA, 1.75 mg/ml albumin and 0.5 mM L-carnitine at pH 7.0. After incubation for 5 min, the reactions were terminated and neutralized. Each of the neutralized perchloric acid extracts (50 μl) was injected into the chromatograph. Peak numbers and chromatographic conditions are as for Fig. 1.

The contamination of CPT II, which could have been caused by mitochondria damaged during cell fractionation [20], as well as any contamination of peroxisomal carnitine octanoyltransferase (COT) [21] was assessed through the suppression of CoASH formation at a high concentration (150 μM) of malonyl-CoA [20]. At this concentration, CPT I was completely inhibited in both fed and starved rats [20], whereas both CPT II [5] and peroxisomal COT [22] were insensitive to malonyl-CoA. The presence of enzymes non-suppres-

sible by the high malonyl-CoA concentration was indicated by the activity of contaminated CPT II and peroxisomal COT. In this experiment, the non-suppressible fraction accounted for $5 \pm 0.4\%$ ($n=6$) of the total activity measured in the absence of malonyl-CoA in both fed and starved animals. The contributions of CPT II and peroxisomal COT were corrected by subtracting the activity of the unsuppressed fractions from the total activity measured in the absence of malonyl-CoA.

Fig. 3 shows the activity of mitochondrial CPT I from the liver of rats starved for 48 h as a function of mitochondrial protein concentration. The values presented in Fig. 3 were corrected by the contribution of fractions not suppressed by the high concentration (150 μM) of malonyl-CoA. The reaction was linear with time for up to 8 min of incubation at 25 °C with 300 μg of mitochondrial protein (data not shown) and with protein concentrations of up to 600 μg of protein per assay mixture for 5-min incubations. Thus, standard assays were carried out for 5-min incubations at 25 °C with ca. 200 μg of mitochondrial protein per assay mixture.

The recoveries of CoASH were estimated by adding 2 nmol of authentic CoASH (100 μl) to 0.9 ml of assay mixture containing 0, 1, 2, 3 and 5 mM dithiothreitol but no palmitoyl-CoA. After 5 min of incubation at 25 °C, the reaction mixture was acidified and then neutralized (pH 2–3) in the same way as in the CPT I assay. Then the neutralized supernatant was allowed to stand at 4 °C. As shown in Table I, dithiothreitol at a final concentration of 3 mM or

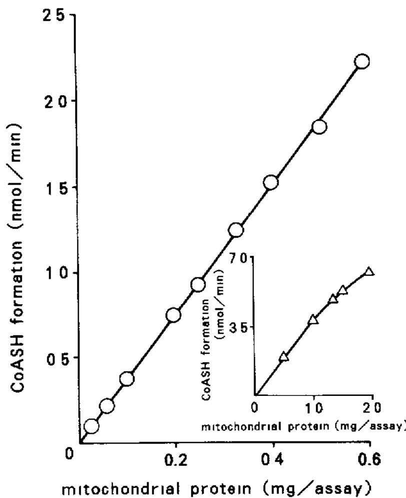

Fig. 3. Effect of mitochondrial protein concentration on CPT I. Assays were performed as described in Experimental, using 80 μM palmitoyl-CoA, 1.75 mg/ml albumin and 0.5 mM L-carnitine at pH 7.0, by incubation at 25 °C for 5 min. Mitochondria were prepared from rats starved for 48 h. All values were corrected by the contribution of non-suppressible fractions by a high concentration (150 μM) of malonyl-CoA.

more is required to preserve CoASH, and so 5 mM dithiothreitol was added in the standard assay procedure. In the presence of dithiothreitol (3 mM or more), the released CoASH (pH 3.0 at 4°C) is stable for at least 12 h.

Table II presents the kinetic parameters of CPT I in the mitochondria from both fed and starved rats, as calculated from linear double-reciprocal plots (Lineweaver–Burk). Since in CPT I assays using albumin the actual concentration of the substrate (the fraction of palmitoyl-CoA unbound to albumin) cannot be determined, the values of both the Michaelis constant (K_M) and the maximum velocity (V_{max}) for palmitoyl-CoA and the dissociation constant for the CPT I–malonyl-CoA complex (K_i) are only apparent values. The palmitoyl-CoA/albumin concentration molar ratio in the assay mixture markedly influenced these kinetic parameters [8,10]. However, valid comparison between the values could still be made, since they were assayed under the same experimental conditions. Starvation increased the apparent V_{max} by 120% without significant alteration of the K_M for palmitoyl-CoA. This result is con-

TABLE I

EFFECT OF DITHIOTHREITOL ON THE RECOVERY OF CoASH

Dithiothreitol (mM)	Recovery (mean ± S.D., $n=4$) (%)				
	0.5 h	1 h	3 h	6 h	12 h
0	52 ± 6.8	20 ± 3.4	4 ± 0.2	3 ± 0.3	3 ± 0.4
1	68 ± 5.9	41 ± 3.1	10 ± 0.9	2 ± 0.2	3 ± 0.4
2	96 ± 6.3	81 ± 7.7	65 ± 5.8	62 ± 6.2	64 ± 5.3
3	95 ± 6.2	90 ± 4.5	97 ± 5.9	96 ± 3.7	93 ± 4.8
5	96 ± 3.1	95 ± 2.1	97 ± 3.3	92 ± 3.3	94 ± 5.0

TABLE II

KINETIC PARAMETERS AND MALONYL-CoA SENSITIVITY OF CPT I IN LIVER MITOCHONDRIA FROM FED RATS AND RATS STARVED FOR 48 h

CPT I activity was assayed, as described in Experimental, using 30–120 μM palmitoyl-CoA, 0.5 mM L-carnitine and 1.75 mg/ml albumin (pH 7.0) at 25°C, in a final volume of 1 ml. K_i was calculated from Dixon plots by using 50 or 80 μM palmitoyl-CoA and 0.5 mM L-carnitine. The malonyl-CoA concentration was varied from 0 to 2.5 μM. Values are means ± S.D. for six animals in each group. All values were corrected by the contribution of non-suppressible fractions by a high concentration (150 μM) of malonyl-CoA.

Mitochondria	V_{max} (nmol/min per mg protein)	K_M for palmitoyl-CoA (μM)	K_i for malonyl-CoA (μM)
Fed group	3.27 ± 0.31	65.79 ± 5.39	0.23 ± 0.03
Starved group	7.19 ± 6.39[a]	77.58 ± 7.81	1.98 ± 0.24[a]

[a] $P < 0.01$ when compared with fed rats (Student's unpaired t-test).

sistent with the findings of Saggerson and Carpenter [7], Cook [9] and Bird and Saggerson [10].

It is well documented that CPT I is more sensitive to inhibition by malonyl-CoA in isolated liver mitochondria from fed rats than in those from starved rats [6,8–10]. The apparent K_i values reported here are nine times as high in rats starved for 48 h than in fed rats (Table II). This increase is in good agreement with a previous report [9].

In conclusion, we have developed a sensitive HPLC assay for the specific determination of CPT I activity in isolated rat liver mitochondria. The precision and accuracy of this assay are comparable with those of the commonly used radioactive assay. When the UV detector sensitivity was set to 0.04 a.u.f.s. as proposed in this experiment, CPT I in rat liver mitochondria can be assayed from only 30 μg of mitochondrial protein per millilitre of assay mixture. Since this sensitivity is ample for studying rat liver and heart mitochondria, the availability of this method will allow studies of CPT I activity and of sensitivity to malonyl-CoA inhibition in various biological materials without the use of radioisotopes.

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