Purification and properties of the soluble carnitine palmitoyltransferase from bovine liver mitochondria

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A new carnitine palmitoyltransferase (CPT) was purified to homogeneity from bovine liver mitochondria which were 96% free of peroxisomal contamination, as judged by catalase and glutamate dehydrogenase activities. The enzyme is easily removed from mitochondria, without the use of detergent. It is monomeric (M_r 63 500), unlike other preparations of CPT from mitochondria, and is most active with myristoyl-CoA and palmitoyl-CoA. The K_m values are between 0.8 and 4 μ M for a range of substrates from hexanoyl-CoA to stearoyl-CoA; these are much lower than values reported for other purified CPT preparations. The K_m for L-carnitine is 185 μ M measured with palmitoyl-CoA, and does not vary greatly with the chain length. This is also lower than the values reported for other CPT preparations, but higher than those cited for the medium-chain transferases. Kinetic and inhibitor studies were consistent with a rapid-equilibrium random-order mechanism. 2-Bromopalmitoyl-CoA, which is an inhibitor of the outer CPT, inhibited the enzyme competitively with palmitoyl-CoA as the variable substrate, when added without preincubation. If the enzyme was preincubated with 2-bromopalmitoyl-CoA and carnitine, the activity did not reappear after gel filtration of the protein. The inhibitor was bound in a 1:1 stoichiometry per subunit of enzyme.

INTRODUCTION

Carnitine palmitoyltransferases (CPTs; EC 2.3.1.21) catalyse the reversible transfer of a long-chain fatty-acyl group from acyl-CoA to L-carnitine. CPT activities are located on both sides of the mitochondrial inner membrane, utilizing the cytosolic or matrix pools of carnitine and CoA. These enzymes, along with the carnitine-acylcarnitine translocase, facilitate the uptake of long-chain fatty acids into the mitochondrial matrix for oxidation (for reviews see Bremer, 1983; Hoppel & Brady, 1985).

Studies of the properties *in situ* of the outer CPT (CPT-A), compared with those of the inner CPT (CPT-B), have revealed a number of differences. CPT-A is more easily removed from rat liver mitochondria by digitonin extraction (Hoppel & Tomec, 1972). In the same tissue, a comparison of CPT kinetics between intact mitochondria and inverted submitochondrial vesicles indicated that CPT-A and CPT-B have different kinetic properties (Brady *et al.*, 1986). In particular, CPT-A is strongly inhibited by malonyl-CoA, but the inhibition is lost when the enzyme is removed from the mitochondrial inner membrane (McGarry *et al.*, 1978; Saggerson, 1982). CPT-B is apparently much less sensitive to malonyl-CoA (McGarry *et al.*, 1978; Brady *et al.*, 1986).

Clarke & Bieber (1981) and Miyazawa *et al.* (1983) have purified CPT from ox heart and rat liver mitochondria respectively. They concluded that one

distinct protein was responsible for all CPT activity in mitochondria. It was proposed that the different properties of CPT-A and CPT-B *in situ* might be due to the different environments experienced by the protein on either side of the mitochondrial inner membrane (Bieber & Farrell, 1983).

In contrast with these reports, West *et al.* (1971) have reported the separation of two pools of CPT activity from ox liver mitochondria. One was readily soluble without detergent and was inactivated by 2-bromopalmitoyl-CoA. The second was tightly bound to membrane material and used 2-bromopalmitoyl-CoA as a substrate. It is known that 2-bromopalmitoyl-CoA acts as an inhibitor of CPT-A in intact mitochondria (Chase & Tubbs, 1972). It was proposed that the easily solubilized enzyme corresponded to CPT-A. This protein was apparently different from the CPT isolated from calf liver mitochondria by Kopec & Fritz (1971), which required a detergent to solubilize it.

Here we report the full purification and properties of the CPT identified by West *et al.* (1971) and Edwards (1973), and demonstrate that it is a monomer with one long-chain acyl-CoA-binding site per monomer. Kinetic and inhibitor studies are presented to compare with the detergent-solubilized, presumably inner, CPT preparations described in the literature (Miyazawa *et al.*, 1983; Clarke & Bieber, 1981; Kopec & Fritz, 1971). The results suggest that the differences between the two enzymes are not solely due to the effect of the membrane, but that they are different molecules.

Abbreviations used: CPT, carnitine palmitoyltransferase; CPT-A and CPT-B, the outer and the inner carnitine palmitoyltransferase (nomenclature of Hoppel & Brady, 1985); CAT, carnitine acetyltransferase; COT, carnitine octanoyltransferase; 4-PDS, 4,4'-dithiobispyridine (4,4'-bispyridine disulphide); aldrithiol).

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EXPERIMENTAL

CPT assay

The problems of the CPT assay have been reviewed (Bieber & Farrell, 1983). In the forward direction, we chose to measure CoASH release by its reaction with 4-PDS (made up in ethanol each week) as described for carnitine acetyltransferase (CAT) (Ramsay & Tubbs, 1975). The assay system (25 °C, 1 ml) contained 25 mM-potassium phosphate, pH 7.5, 125 μ M-4-PDS, acyl-CoA as indicated (e.g. 25 μ M-palmitoyl-CoA), enzyme and 2 mM-L-carnitine. In crude samples the hydrolase rate was determined, and then L-carnitine was added to initiate the reaction. The molar absorption coefficient for the 4-thiopyridone is 19600 M⁻¹·cm⁻¹ at 324 nm. It is important to note that CPT activity is competitively inhibited by both Hepes and Mops, perhaps because, like carnitine, they are zwitterions. The K_i for Hepes was 55 mM, and for Mops was estimated to be 65 mM. The ionic strength was kept constant by addition of NaCl.

The reverse reaction was measured at 232 nm. The assay contained, in 1 ml, 20 mM-potassium phosphate, pH 7.4, 5–100 μ M-palmitoyl-L-carnitine, 0.2 mM-CoASH and 2 μ g of enzyme/ml. Both reactions were followed with a Uvikon 810 spectrophotometer (Kontron Instruments).

Carnitine solutions (neutralized with KOH) were standardized by using acetyl-CoA and CAT (Sigma) in the 4-PDS assay. Solutions of acyl-CoA were measured by the reaction with 4-PDS of the CoASH released after alkaline hydrolysis.

Other enzymes

Carnitine octanoyltransferase (COT) was measured as described above, but with 0.2 mM-octanoyl-CoA, and CAT was assayed with 0.2 mM-acetyl-CoA. Glutamate dehydrogenase, catalase and urate oxidase were measured by standard methods (Bergmeyer, 1974).

Protein was measured by the biuret method (Gornall *et al.*, 1949) for crude samples and by the Lowry *et al.* (1951) method for pure samples.

Solubilization and purification of CPT

These were based on the procedures of West *et al.* (1971) and Edwards (1973).

Bovine liver mitochondria were prepared by the method of Kearney *et al.* (1971) and frozen for up to 4 months. The purity of the mitochondrial preparations is discussed below. The mitochondria (50 g) were almost thawed and blended vigorously with 2 vol. of cold glass-distilled water for 2 min. The pH was adjusted to 5.2 with 20% (v/v) acetic acid and the homogenate stirred for 20 min at 4 °C. After centrifuging at 20000 *g* for 20 min, the supernatant contained 15–25% of the total CPT activity. The enzyme was precipitated with 55%-satd. (NH₄)₂SO₄, resuspended in 10 mM-potassium phosphate, pH 7.0, and dialysed overnight against running tap water. A considerable precipitate formed, but it contained no CPT activity and it was removed by centrifugation at 20000 *g* for 10 min.

The supernatant was adjusted to 5 mM-potassium phosphate by addition of 1 M-potassium phosphate, pH 7.5, and to pH 7.5 with KOH. It was applied to

a DEAE-cellulose column (20 cm × 6.5 cm), which was washed with 10 mM-potassium phosphate, adjusted to pH 7.5 with KOH, until no further protein was eluted. The CPT activity was eluted with 20 mM-potassium phosphate, pH 7.5. Acyl-CoA hydrolase activity is removed by this step. The yield was only about 60%, because residual membrane-bound enzyme was washed straight through the column. The active fractions were applied directly to a column (25 cm × 3.5 cm) of Blue Sepharose CL-6B (Sigma) equilibrated with 20 mM-potassium phosphate, pH 7.5. The column was washed with 300 ml of starting buffer, then with the buffer containing 80 mM-KCl until the A_{280} of the eluate returned to the baseline (about 400 ml). The CPT activity was eluted with a gradient of 80–400 mM-KCl in 20 mM-potassium phosphate, pH 7.5 (900 ml each). The pooled fractions were concentrated by ultrafiltration (Amicon PM30) with gentle stirring. The preparation was diluted 5-fold and applied to a small column (18 cm × 1.5 cm) of Blue Sepharose CL-6B equilibrated with 20 mM-potassium phosphate, pH 7.5. After washing with buffer containing 50 mM-KCl, the CPT was affinity-eluted with octanoyl-CoA (10 mg in 100 ml of washing buffer). This elution is slightly variable, depending on the amount of hydroxymethylglutaryl-CoA synthase carried through from earlier stages. Octanoyl-CoA elutes less of this enzyme than does CoA. [If pure enzyme is not obtained at this stage, a final chromatofocusing step can be used, with a column (1 cm × 10 cm) of PBE 94 (Pharmacia) equilibrated with Polybuffer 74 (Pharmacia) diluted 25-fold and adjusted to pH 5 with HCl. CPT is eluted at pH 6, its pI.] The pooled fractions were concentrated to 1 mg/ml and dialysed against 20 mM-potassium phosphate, adjusted to pH 7.5 with KOH, containing 0.2 mM-dithioerythritol, for 3 h. The enzyme is stable for years when stored in 20% glycerol/0.2 mM-dithioerythritol at –20 °C.

The purity of the enzyme was assessed by SDS/polyacrylamide-gel electrophoresis. The specific activity of the purest preparation in the 4-PDS assay with 20 μ M-palmitoyl-CoA and 2 mM-L-carnitine was 42 μ mol/min per mg.

Separation of mitochondria and peroxisomes

Mitochondrial and peroxisomal fractions were isolated essentially as described by Fleischer & Kervina (1974). Fresh ox liver (50 g) was finely chopped in 150 ml of 0.25 M-sucrose/10 mM-Hepes, pH 7.5, at 4 °C (sucrose medium), gently homogenized by two full passes in a Dounce homogenizer, and filtered through six layers of muslin. After sedimentation at 1200 *g* for 10 min, the pellet was resuspended in sucrose medium (A). The supernatant was filtered through four layers of muslin and centrifuged at 39000 *g* for 10 min. The pellet was resuspended in sucrose medium containing 0.6% ethanol (B). Both samples (A and B) were run on sucrose density gradients containing 5% dextran and 0.1% ethanol as described by Fleischer & Kervina (1974) for 1 h only at 200000 *g* (r_{av} = 10.9 cm) and harvested immediately at 4 °C. Fractions were assayed for protein and, after 2-fold dilution with 20 mM-potassium phosphate, pH 7.5, containing 0.1% Triton X-100, for CPT, COT, CAT, glutamate dehydrogenase (mitochondrial marker) and catalase (peroxisomal marker).

Determination of M_r

Subunit M_r was determined by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970). Native M_r was determined by gel filtration on Sephadex G-150, by non-denaturing gel electrophoresis (Hedrick & Smith, 1968), by sedimentation on sucrose gradients (Martin & Ames, 1961), and by h.p.l.c. on a Spherogel TSK 3000 column.

Materials

Column materials were purchased from Whatman, except for Sepharose CL-6B, which came from Pharmacia. 4-PDS was obtained from Aldrich. The acyl-CoA substrates and other biochemicals (analytical grade) came from Sigma. *N*-Trimethylamino-undecanoyl-DL-carnitine (Ramsay & Tubbs, 1986) and 2-bromopalmitoyl-CoA (Chase & Tubbs, 1972) were synthesized as described in the references.

RESULTS AND DISCUSSION

Purification of the soluble CPT

The ease of solubilization of this enzyme (15–25% of the total CPT activity) distinguishes it from other mitochondrial preparations of CPT (Kopeck & Fritz, 1971; Clarke & Bieber, 1981; Miyazawa *et al.*, 1983). Only peroxisomal COT is as easily solubilized from whole tissue (Miyazawa *et al.*, 1983), but contaminating peroxisomes were unlikely to be the source of this CPT (see below). CPT is released from ox liver mitochondria by water extraction after a freeze-thaw cycle, and attempts to re-attach it to depleted mitochondrial membranes were unsuccessful. The earlier steps in the purification (see Table 1) were based on previous work (West *et al.*, 1971; Edwards, 1973), but affinity elution and sometimes chromatofocusing were required to obtain homogeneous enzyme. The purification is shown in Table 1. The enzyme had no impurities detectable by SDS/polyacrylamide-gel electrophoresis (Fig. 1, lanes 7 and 8).

It was important to establish that the enzyme was mitochondrial, and not peroxisomal, in origin if it were to be demonstrated to be CPT-A. The activities of CPT,

Fig. 1. SDS/polyacrylamide-gel electrophoresis of CPT during and after its purification from ox liver mitochondria

Tracks 1 and 6, M_r markers (phosphorylase *b*, 92500; bovine serum albumin, 66200; ovalbumin, 45000; carbonic anhydrase, 31000; soya-bean trypsin inhibitor, 21500; lysozyme, 14400; the mixture was a low- M_r marker kit from Bio-Rad); tracks 2 and 3, 2 and 4 μ g, pooled fractions after DEAE-cellulose step; tracks 4 and 5, 2 and 5 μ g, after salt elution from the large Blue Sepharose column; tracks 7 and 8, 1 and 3 μ g, purified enzyme after affinity elution.

Table 1. Purification of CPT from ox liver mitochondria

Step	Total activity (units)	Specific activity (units/mg)	Yield (%)
Water homogenate*	4050	0.058	—
Acid supernatant	571	0.040	100
Acid pellet	3050	0.064	—
Dialysed after $(\text{NH}_4)_2\text{SO}_4$	441	0.065	77
DEAE-cellulose	242	0.610	42
Large Blue Sepharose†	161	5.43	28
Affinity elution†	95	43.3	16.6

* Assay after treatment with 0.1% Triton.

† Losses of total units of activity occur mainly during the concentration procedures after these steps. Column yields are generally 85–90%.

COT, CAT and marker enzymes were measured after separation of the organelles from whole liver homogenates on sucrose gradients (Fig. 2). Ratios of the averaged specific activities of the three peak fractions for peroxisomes (Fig. 2a) and mitochondria (Fig. 2b) were compared (Table 2). The results show that CAT is completely mitochondrial. COT is enhanced in peroxisomes, but not as much as catalase, whereas in the mitochondrial peak the enhancement is greater than for catalase. This distribution is consistent with the presence of COT in peroxisomes and the contribution to the total COT activity by mitochondrial CPT. The CPT ratios do not mimic COT. Most CPT activity is mitochondrial, and the very slight enhancement in peroxisomes above the peroxisomal/mitochondrial ratio for glutamate dehydrogenase is probably due to cross-reactivity of COT. The averaged ratio of COT/CPT activity in mitochondria is 0.5 in both Figs. 2(a) and 2(b), and in peroxisomes is 0.98 (Fig. 2a). The latter ratio is much less than the 4-fold excess of COT activity found in mouse liver peroxisomes (Farrell *et al.*, 1984) or the 10-fold excess found in rat liver peroxisomes (Miyazawa *et al.*, 1983), but is clearly different from the mitochondrial ratio.

To eliminate further the peroxisomes as a source of the soluble CPT, one routine preparation was monitored by assaying samples at each step immediately for cytochrome oxidase, glutamate dehydrogenase, catalase, urate oxidase and *L*- α -hydroxyacid oxidase. Portions of each sample were promptly treated with no addition, or 0.1% ethanol (to protect catalase activity), or 0.1% Triton

Fig. 2. Carnitine acyltransferase activities from ox liver in peroxisomes and mitochondria after separation by sucrose-density-gradient centrifugation

The methods and assays are described in the Experimental section. (a) Mitochondrial/perixosomal fraction; (b) nuclear/mitochondrial fraction. The top panels show the acyltransferase activities, CAT ($\circ$), COT (Δ) and CPT ($\square$). The bottom panels show protein ($\circ$), the peroxisomal marker catalase (Δ), and the mitochondrial marker glutamate dehydrogenase ($\square$).

X-100 (to lyse particles), and the highest activity measured was used in compiling the recovery and contamination of the mitochondria. The results were comparable with data obtained by Leighton *et al.* (1968) for a mitochondria preparation in which they estimated about 1.5% of the protein came from peroxisomes (results not shown). Certainly peroxisomal contamination of the mitochondria was insufficient to account for 20% of the CPT activity.

Properties of CPT

The properties of the previously purified CPT preparations have been reviewed (Hoppel & Brady, 1985). For this easily solubilized CPT, the subunit M_r ($63\,500 \pm 800$) is lower than that obtained with detergent-solubilized preparations (e.g. Clarke & Bieber, 1981; Miyazawa *et al.*, 1983), but similar to the peroxisomal COT from rat liver (Miyazawa *et al.*, 1983). The native

M_r was examined by gel electrophoresis, column chromatography, h.p.l.c. and sucrose-gradient sedimentation. In all cases the activity was recovered in monomeric form, unlike the high- M_r detergent-solubilized enzymes (Bergstrom & Reitz, 1980; Clarke & Bieber, 1981; Miyazawa *et al.*, 1983). The pI is low (pH 6.0), which distinguishes this preparation from the detergent-solubilized CPT preparation of Clarke & Bieber (1981), which had a pI of around 8. The amino acid composition (Table 3) is similar to that found by Clarke & Bieber (1981), except that we find twice as much glycine.

The pH-dependence of the enzyme activity in the 4-PDS assay was examined. At fixed palmitoyl-CoA ($25\,\mu\text{M}$) and varied carnitine concentrations, V_{max} does not change between pH 6.0 and 8.5, in contrast with the decrease observed for ox heart CPT-B (Fiol & Bieber, 1984). K_m for carnitine increases below pH 7.0, as observed with other preparations (Fiol & Bieber, 1984;

Table 2. Relative activities of the peroxisomal and mitochondrial peak fractions from density-gradient centrifugation

The relative activity of an enzyme in one fraction is its specific activity there divided by the specific activity of the same enzyme in the contaminating fraction; (a) and (b) correspond to the gradients in Fig. 2. Abbreviation: GDH, glutamate dehydrogenase.

		Relative activity				
		GDH	Catalase	CAT	COT	CPT
(a)	Peroxisomes	0.53	2.0	0.66	1.62	0.81
	Mitochondria	1.88	0.5	1.5	0.62	1.23
(b)	Peroxisomes	0.53	2.21	0.48	1.19	0.59
	Mitochondria	1.88	0.45	2.08	0.84	1.68

Mills *et al.*, 1984). Salt concentration has only a small effect on activity, in agreement with the lack of effect on digitonin-released CPT activity (Wood, 1973). However, zwitterionic buffers, such as Hepes and Mops, competitively inhibit the enzyme. The apparent K_i values were 55 mM for Hepes and 65 mM for Mops.

The K_m values for the substrates at pH 7.4 in the forward direction were $182 \pm 0.14 \mu\text{M}$ for L-carnitine and $0.82 \pm 0.30 \mu\text{M}$ for palmitoyl-CoA, and in the reverse direction were $6.7 \pm 0.9 \mu\text{M}$ for palmitoyl-L-carnitine and $21 \pm 0.5 \mu\text{M}$ for CoASH (see Table 4). The ratio of the V_{max} values for octanoyl-CoA/palmitoyl-CoA was 0.57, similar to that observed for CPT-B from rat liver mitochondria (0.5; Miyazawa *et al.*, 1983), but different from that for calf liver (0.015; Kopec & Fritz, 1973) and ox heart (1.13; Clarke & Bieber, 1981) enzyme. It should be noted that the assay conditions, especially for palmitoyl-CoA, affect these ratios enormously (see Miyazawa *et al.*, 1983), so the ratios from the different laboratories should be compared with caution.

Kinetic studies

The substrate specificity of soluble CPT is shown in Table 5. The pattern of the apparent V_{max} values is similar to that found by Miyazawa *et al.* (1983), but the peak value occurs at a longer chain length than was observed by Clarke & Bieber (1981) for CPT from ox heart. The K_m values for all acyl-CoA substrates except acetyl-CoA are low, indicating that all chain lengths from C_4 up will compete for the enzyme. The apparent K_m for L-carnitine was only slightly affected by the chain length of the acyl-CoA substrate, and was slightly lower than most reported values [see Hoppel & Brady (1985) for a summary Table], except for that reported for rat liver by Bird *et al.* (1985), but much higher than the values of 20–40 μM reported for COT enzymes (Miyazawa *et al.*, 1983; Farrell *et al.*, 1984).

Table 4 summarizes the kinetic constants for the forward and backward reactions in the absence and presence of Tween 20 or bovine serum albumin, additions often used to minimize problems arising from the low micelle concentration of the longer-chain acyl-CoA substrates and the K_m values. With bovine serum albumin present, the true free concentration of palmitoyl-CoA is unknown, and, at least for CPT-A

Fig. 3. Rate and stoichiometry of the irreversible inhibition of purified CPT by 2-bromopalmitoyl-CoA

(a) Inactivation of CPT at 30 °C. Individual incubations contained 20 mM-potassium phosphate, pH 7.5, 2 mM-L-carnitine and CPT (2 $\mu\text{g}/\text{ml}$), with (○) or without (●) 2.2 μM -2-bromopalmitoyl-CoA. At the indicated times, activity was measured after addition of 125 μM -4-PDS and 15 μM -palmitoyl-CoA. The units of v were $\mu\text{mol}/\text{min}$ per mg. (b) Spectra of CPT with (a, —) and without (b, ---) 2-bromopalmitoyl-CoA. CPT (3.2 nmol; 1.06 mg/ml) was incubated for 1 h at 30 °C with L-carnitine (6 nmol) with or without 2-bromopalmitoyl-CoA (6 nmol). The samples were chilled, passed through Sephadex G-50 (fine grade) and diluted to 3 times the original volume. The spectrum of each sample (a or b) was recorded by using a diode-array spectrophotometer (Hewlett-Packard HP 84519). Spectrum c (· · · · ·) is the difference spectrum, and is identical in form with the spectrum for 2-bromopalmitoyl-CoA alone.

in situ, the palmitoyl-CoA/albumin ratio affects the apparent K_m for L-carnitine (Edwards *et al.*, 1985). For the purified enzyme, with a constant albumin/palmitoyl-CoA ratio, the kinetic constants were only slightly affected (Table 4). The dramatic effect of Tween 20 on detergent-solubilized CPT from rat liver has been described (Miyazawa *et al.*, 1983). Its addition, therefore, rather complicates the interpretation of the data, but in its absence the K_m for palmitoyl-CoA is so low that the total absorbance change for concentrations in the required range is very small.

Table 3. Amino acid composition of ox liver soluble CPT

The amino acid analysis was performed in the laboratory of Dr. A. J. Smith (University of California, Davis). The amounts of each amino acid are expressed as mol/100 mol of total amino acid detected after 24 h hydrolysis, with or without performic acid oxidation. The minimum M_r based on cysteine was found to be 8220, and the molar composition was thus calculated by multiplying the amounts of each amino acid by 8. Abbreviation: n.d., not determined.

Amino acid	Composition	
	(%)	(mol/mol of subunit)
Asx	8.6	51
Thr	3.1	18
Ser	7.1	42
Glx	13.9	82
Pro	5.5	32
Gly	11.7	71
Ala	6.8	40
Val	6.6	39
Met	1.8	10
Ile	3.2	19
Leu	10.0	60
Tyr	3.5	20
Phe	4.0	24
His	2.6	16
Lys	5.6	32
Arg	4.7	28
Cys	1.3	8
Trp	n.d.	n.d.

Inhibitors

Table 6 summarizes the kinetic effects of inhibitors on the forward reaction of CPT in the absence and presence of Tween 20. The inhibition patterns are consistent with the rapid-equilibrium random-order mechanism suggested by kinetic studies. When the inhibitor has a long acyl chain, mixed inhibition is observed, consistent with the proximity of the two substrate sites proposed by Chase & Tubbs (1972). 2-Bromopalmitoyl-CoA was an excellent competitive inhibitor (when added without preincubation), consistent with its similarity to palmitoyl-CoA and with the concentration required to cause 50% of the

Table 5. Substrate specificity for CPT assayed in the forward direction

Acyl-CoA substrate chain length	Relative V_{max} (%)	K_m (acyl-CoA)* (μM)	K_m (L-carnitine)† (μM)
C ₂	0.6	22.0	
C ₄	22.0	5.1	
C ₆	45.0	1.8	
C ₈	57.0	0.8	108
C ₁₀	69.0	0.9	
C ₁₂	74.0	4.0	120
C ₁₄	98.0	1.2	
C ₁₆	100.0	0.8	185
C ₁₈	79.0	1.7	

* L-Carnitine concentration was 2 mM.

† Acyl-CoA concentrations were: C₁₆, 20 μM ; C₁₂, 50 μM , C₈, 100 μM .

maximum inhibition of CPT-A in intact rat liver mitochondria (0.31 μM ; Edwards *et al.*, 1985). 2-Bromoacetyl-CoA was a very poor inhibitor of the purified CPT (as also found by Miyazawa *et al.*, 1983), consistent with the observation that solubilization causes loss of its malonyl-CoA-like effect observed in intact rat liver mitochondria (Edwards *et al.*, 1985). Malonyl-CoA is a competitive inhibitor of the purified enzyme when assayed at palmitoyl-CoA concentrations below the critical micelle concentration. There is no evidence for a regulating malonyl-CoA-sensitive site in the purified enzyme.

Certain inhibitors can be used to distinguish this CPT preparation from peroxisomal COT and from CPT-B. Peroxisomal COT isolated from mouse liver was not inactivated by 5,5'-dithiobis-(2-nitrobenzoate) (Farrell *et al.*, 1984). In our preparation, CPT is completely inactivated by incubation with 125 μM -4-PDS under the conditions described for the assay, with $t_{1/2}$ of 10 min. Substrates (25 μM -palmitoyl-CoA and/or 2 mM-L-carnitine) protect against inactivation (results not shown). Irreversible inhibition of CPT-A by 2-bromoacyl substrates has been described (Chase & Tubbs, 1972; Edwards *et al.*, 1985). The irreversible inhibition requires incubation of the enzyme with the inhibitor in the presence of L-carnitine and occurs only with CPT-A

Table 4. Kinetic constants of soluble CPT in the absence and presence of Tween 20 or albumin

Abbreviation: n.d., not determined

	Forward*			Reverse†		
	K_m (μM)		V_{max} ($\mu mol/min$ per mg)	Apparent K_m (μM)		Apparent V_{max} ($\mu mol/min$ per mg)
	L-Carnitine	C ₁₆ -CoA		C ₁₆ -carnitine	CoA	
No additions	182	0.8	58	6.7	21	31
0.4% Tween 20	191	61.7	66	138	n.d.	21
Bovine serum albumin (40 mg/ μmol of palmitoyl-CoA)	200	1.0	60	n.d.	n.d.	n.d.

* 4-PDS assay, with palmitoyl-CoA as the substrate.

† Assay at 232 nm.

Table 6. Kinetic effects of inhibitors of CPT on the forward reaction

The 4-PDS assay is described in the Experimental section. The fixed substrate concentrations were 25 μ M-palmitoyl-CoA or 2 mM-L-carnitine. The inhibition patterns were competitive (C), non-competitive (NC) or mixed (M): n.d., not determined.

Inhibitor	Varied substrate . . .	Apparent K_i (mM)	
		Palmitoyl-CoA	L-Carnitine
<i>(a)</i> Without detergent			
2-Bromopalmitoyl-CoA		0.66×10^{-3} (C)	n.d.
2-Bromoacetyl-CoA		0.90 (C)	n.d.
Malonyl-CoA		0.17 (C)	n.d.
11-Trimethylamino-undecanoyl-DL-carnitine		2.0 (M)	2.0 (C)
<i>(b)</i> With 0.4% Tween 20			
2-Bromopalmitoyl-CoA		0.2×10^{-3} (C)	0.1×10^{-2} (M)
Deoxycarnitine		12.0 (NC)	12 (C)

(Chase & Tubbs, 1972). Carnitine-dependent inactivation by 2-bromopalmitoyl-CoA is now an established method of distinguishing between CPT-A and CPT-B (Bremer, 1983). Miyazawa *et al.* (1983) have reported that peroxisomal COT is also inactivated by 2-bromopalmitoyl-CoA, so CPT-A and peroxisomal COT resemble each other in this respect. The mechanism is assumed to be via the formation of the *S*-carboxypalmitoyl-CoA ester of L-carnitine, in a manner analogous to the inhibition of CAT by 2-bromoacetyl-CoA and carnitine (Chase & Tubbs, 1969; Tubbs *et al.*, 1980). During the incubation, CPT-A catalyses the formation of 2-bromopalmitoyl-L-carnitine and CoA. The terminal thiol group of CoA then alkylates the bromopalmitoyl-carnitine, with the loss of bromine. The resulting adduct is very tightly bound to the active site. Fig. 3(a) shows the time course for the inactivation of purified CPT by 2-bromopalmitoyl-CoA in the presence of L-carnitine. The apparent first-order rate constant at 30 °C was 0.023 s^{-1} . To establish that the inhibition was irreversible, CPT was incubated with a 2-fold excess of 2-bromopalmitoyl-CoA and carnitine for 1 h at 30 °C. Excess inhibitor was removed by passing the enzyme through Sephadex G-50, and the enzyme was diluted to 3 times the original volume. There was no re-appearance of activity, even after 8 days incubation on ice, and the residual activity (14% after the removal of the inhibitor) declined in proportion to the loss of activity in the control sample (which retained only 30% activity after 8 days). In the same experiment, the stoichiometry for the irreversible inhibition was measured (Fig. 3b). The amount of 2-bromopalmitoyl-CoA bound to the enzyme at the start of the experiment was assessed by the difference spectrum of the inhibited enzyme minus that of the control enzyme. The absorption coefficient for 2-bromopalmitoyl-CoA at 260 nm is $0.036 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Assuming that the 14% residual activity represented enzyme without bound inhibitor, a total of 3.32 nmol of 2-bromopalmitoyl-CoA was bound to 2.84 nmol of CPT (based on the M_r of 63500), a ratio of 1.17 mol of inhibitor/mol of enzyme. This stoichiometry suggests one inhibitor-binding site per monomer, consistent with the model described above. The bound inhibitor could not be extracted from the enzyme by either buffer or ether, even after precipitation of the protein, so covalent binding cannot be ruled out.

This study on completely purified, soluble, CPT from liver mitochondria confirms the observations of West *et al.* (1971) and Edwards (1973) on the partially purified enzyme. Although this enzyme resembles the COT isolated by Miyazawa *et al.* (1983) in its lower M_r and sensitivity to 2-bromopalmitoyl-CoA, it is probably not peroxisomal, and its substrate specificity and sensitivity to thiol reagents is that of their CPT from rat liver mitochondria and of CPT isolated from ox heart mitochondria (Clarke & Bieber, 1981). We consider that its properties both in purified form (the present paper) and *in situ* (West *et al.*, 1971; Edwards *et al.*, 1985) suggest that it is indeed distinct from the tightly membrane-bound CPT-B (Edwards, 1973; Bergstrom & Reitz, 1980; Clarke & Bieber, 1981). Confirmation must await immunological studies and the direct kinetic comparison of the purified enzymes.

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REFERENCES

- Bergmeyer, H. U. (1974) *Methods of Enzymatic Analysis*, Academic Press, New York
- Bergstrom, J. D. & Reitz, R. C. (1980) *Arch. Biochem. Biophys.* **204**, 71–79
- Bieber, L. L. & Farrell, S. (1983) *Enzymes* 3rd Ed. **16**, 627–644
- Bird, M. I., Munday, L. A., Saggerson, E. D. & Clark, J. B. (1985) *Biochem. J.* **226**, 323–330
- Brady, L. J., Hoppel, C. L. & Brady, P. S. (1986) *Biochem. J.* **233**, 427–433
- Bremer, J. (1983) *Physiol. Rev.* **63**, 1420–1480
- Chase, J. F. A. & Tubbs, P. K. (1969) *Biochem. J.* **111**, 225–235
- Chase, J. F. A. & Tubbs, P. K. (1972) *Biochem. J.* **129**, 55–65
- Clarke, P. R. H. & Bieber, L. L. (1981) *J. Biol. Chem.* **256**, 9861–9868
- Edwards, M. R. (1973) Ph.D. Thesis, University of Cambridge
- Edwards, M. R., Bird, M. I. & Saggerson, E. D. (1985) *Biochem. J.* **230**, 169–179
- Farrell, S. O., Fiol, C. J., Reddy, J. K. & Bieber, L. L. (1984) *J. Biol. Chem.* **259**, 13089–13095
- Fiol, C. J. & Bieber, L. L. (1984) *J. Biol. Chem.* **259**, 13084–13088
- Fleischer, S. & Kervina, M. (1974) *Methods Enzymol.* **31**, 6–41
- Gornall, A. C., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766

- Hedrick, J. L. & Smith, A. J. (1968) *Arch. Biochem. Biophys.* **126**, 155–164
- Hoppel, C. L. & Brady, L. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A. N.), vol. 2, 2nd edn., pp. 139–169, Plenum Press, New York
- Hoppel, C. L. & Tomec, R. J. (1972) *J. Biol. Chem.* **247**, 832–841
- Kearney, E. B., Salach, J. I., Walker, W. H., Seng, R. L., Kenney, W., Zeszotek, E. & Singer, T. P. (1971) *Eur. J. Biochem.* **24**, 321–327
- Kopec, B. & Fritz, I. B. (1971) *Can. J. Biochem.* **49**, 941–948
- Kopec, B. & Fritz, I. B. (1973) *J. Biol. Chem.* **248**, 4069–4074
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J. W., Fowler, S. & DeDuve, C. (1968) *J. Cell Biol.* **37**, 482–513
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372–1379
- McGarry, J. D., Leatherman, G. F. & Foster, D. W. (1978) *J. Biol. Chem.* **253**, 4128–4136
- Mills, S. E., Foster, D. W. & McGarry, J. D. (1984) *Biochem. J.* **219**, 601–608
- Miyazawa, S., Ozasa, H., Osumi, T. & Hashimoto, T. (1983) *J. Biochem. (Tokyo)* **94**, 529–542
- Ramsay, R. R. & Tubbs, P. K. (1975) *FEBS Lett.* **45**, 21–25
- Ramsay, R. R. & Tubbs, P. K. (1986) *Biochem. J.* **235**, 297–299
- Saggerson, E. D. (1982) *Biochem. J.* **202**, 397–405
- Tubbs, P. K., Edwards, M. R. & Ramsay, R. R. (1980) in *Carnitine Biosynthesis, Metabolism and Functions* (Frenkel, R. A. & McGarry, J. D., eds.), pp. 207–218, Academic Press, New York
- West, D. W., Chase, J. F. A. & Tubbs, P. K. (1971) *Biochem. Biophys. Res. Commun.* **47**, 912–918
- Wood, J. M. (1973) *Biochemistry* **12**, 5268–5271

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