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Characterization of hepatic carnitine palmitoyltransferase

Use of bromoacyl derivatives and antibodies

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Carnitine palmitoyltransferase (CPT) is a mitochondrial-inner-membrane enzyme, with activities located on both the outer and inner sides of the membrane. The inhibition of CPT by bromopalmitate derivatives was studied in intact hepatic mitochondria (representing CPT-A activity, the outer enzyme), in inverted submitochondrial vesicles (representing CPT-B, the inner enzyme), and in purified hepatic CPT. Bromopalmitoyl-CoA had an I_{50} (concentration giving 50% inhibition of CPT activity) of 0.63 ± 0.08 μM in intact mitochondria and 2.44 ± 0.86 μM in inverted vesicles. Preincubation of mitochondria with bromopalmitoyl-CoA decreased $V_{max.}$ for both CPT-A and CPT-B. Sonication decreased sensitivity to bromopalmitoyl-CoA, and solubilization with Triton abolished sensitivity at the concentrations used (0–10 μM). Purified CPT had a bromopalmitoyl-CoA I_{50} of 353 μM in aqueous buffer, 67 μM in 20% dimethyl sulphoxide, 45 μM in phosphatidylcholine liposomes and 26 μM in cardiolipin liposomes. Increasing [carnitine] at constant bromopalmitoyl-CoA concentrations or increasing [bromopalmitoyl-CoA] in the preincubation resulted in increased inhibition of purified CPT. 2-Tetradecylglycidyl-CoA and malonyl-CoA did not offer measurable protection against bromopalmitoyl-CoA inhibition of the purified CPT, suggesting a different site of interaction of bromopalmitoyl-CoA with CPT. The data suggest that the sensitivity of CPT to bromopalmitoyl-CoA may be modulated by membrane environment and assay conditions.

INTRODUCTION

The short-term regulation of carnitine palmitoyltransferase (CPT) was originally described as simple competitive inhibition of palmitoyl-CoA binding by malonyl-CoA (reviewed by McGarry & Foster, 1980). More recent evidence indicates that this system is more complex, and that malonyl-CoA may compete with palmitoyl-CoA for a regulatory site other than the catalytic site (Bird & Saggerson, 1984; Mills *et al.*, 1984; Grantham & Zammit, 1986). Thus the question of allosteric regulation of CPT has been raised (Bieber & Fiol, 1986; Grantham & Zammit, 1986).

Brominated derivatives of acetyl-CoA have been proposed to bind to the active site of carnitine acetyltransferase (CAT), and a mechanism involving formation of an *S*-carboxymethyl-CoA carnitine ester has been proposed (Chase & Tubbs, 1969). Grantham & Zammit (1986) have proposed that this mechanism is also operative for CPT of intact mitochondria, where an *S*-carboxypalmitoyl-CoA carnitine ester would result, and Saggerson & Carpenter (1986) have shown increased bromopalmitoyl-CoA sensitivity when bromopalmitoyl-CoA and L-carnitine are added before initiation of the reaction. Previous studies of bromopalmitoyl- and bromostearoyl-CoA inhibition of CPT have left some question as to whether both inner (CPT-B) and outer (CPT-A) enzymes are inhibited, and under what conditions inhibition is expressed.

Yates & Garland (1970) and Chase & Tubbs (1972) have determined that 2-bromopalmitoyl-CoA inhibits CPT and fatty acid oxidation, but that the effect depended on mitochondrial integrity and the presence of cofactors. Edwards *et al.* (1985) have used bromopalmitoyl-CoA inhibition to differentiate the malonyl-CoA-binding site from the acyl-CoA- and bromopalmitoyl-CoA-binding site in intact mitochondria, and Saggerson & Carpenter (1986) have found that bromopalmitoyl-CoA sensitivity differs in various tissues. Grantham & Zammit (1986) have used bromopalmitoyl-CoA plus carnitine to block the presumed CPT-A active site, and concluded that malonyl-CoA binding was not affected. West *et al.* (1971) and Hoppel & Tomec (1972) observed bromopalmitoyl-CoA inhibition of supernatant CPT released by sonication and centrifugation, but not of CPT associated with the pellet after centrifugation. Miyazawa *et al.* (1983) did not observe bromopalmitoyl-CoA inhibition of purified rat liver CPT derived from the mitochondrial-inner-membrane fraction after freeze–thawing and centrifugation at 144000 *g* for 40 min, but Hoppel *et al.* (1986) found bromopalmitoyl-CoA inhibition of purified ox liver CPT derived from the supernatant after sonication in hypo-osmotic phosphate buffer and subsequent centrifugation.

These disparate data focus on two questions: (1) are there two CPT proteins, or are differences in inhibition by bromopalmitate a function of differing membrane

Abbreviations used: CPT, carnitine palmitoyltransferase (EC 2.3.1.21); CPT-A and CPT-B, the CPT activity in the outer and the inner surface of the mitochondrial inner membrane respectively [nomenclature described by Hoppel (1982) and used by Kiorpes *et al.* (1984); other authors have referred to CPT-A as CPT^o, outer transferase and CPT-I, and to CPT-B as CPT^i, inner transferase and CPT-II]; CAT, carnitine acetyltransferase; Me_2SO, dimethyl sulphoxide; I_{50}, concentration giving 50% inhibition.

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environments, coupled with specific assay conditions? and (2) does bromopalmitoyl-CoA bind at the CPT active site or is an alternative site, such as that proposed for malonyl-CoA, likely? The present studies were designed to examine these questions using intact mitochondria, inverted submitochondrial vesicles and purified CPT from rat liver.

EXPERIMENTAL

Animals

Sprague–Dawley rats, weighing 100–300 g, were obtained from the Washington State University colony. The rats had access to laboratory chow and water *ad libitum* before the experiments. For these experiments, rats were either fed or starved for 24 h.

Mitochondria and inverted submitochondrial vesicles

Mitochondria were prepared from rat liver by differential centrifugation after homogenization with a loose-fitting Teflon pestle. This procedure has been described in detail (Hoppel *et al.*, 1979). Inverted submitochondrial vesicles were prepared from the fresh mitochondria by nitrogen compression/decompression (Fleischer *et al.*, 1974). Additional purification of the inverted vesicles was accomplished by cytochrome *c*–Sepharose affinity separation (Godinot & Gautheron, 1979), with the modification that vesicles were treated in batches. These procedures have been described in detail previously (Brady *et al.*, 1985; P. S. Brady & L. J. Brady, 1986).

In some experiments, mitochondria were solubilized in 0.05% Triton X-100. In others, mitochondria were sonicated on ice in 20 mM-potassium phosphate/1 mM-EDTA, pH 7.4, for 3 × 15 s with 20 s cooling periods between sonication. For some of the sonication studies, the sonicated mitochondria were centrifuged at 100000 *g* for 1 h, and the supernatant was removed by aspiration. The pellet was resuspended in the same buffer as for sonication with or without detergent for subsequent study as noted. This procedure has been suggested to remove CPT-A into the supernatant, while CPT-B remains in the pellet (Hoppel, 1982).

In one series of studies, intact mitochondria were incubated for 5 min at 25 °C with 50 μM-bromopalmitoyl-CoA with or without 5 mM-L-carnitine in the same buffer as used to isolate mitochondria (220 mM-mannitol/70 mM-sucrose/5 mM-Mops; 'isolation buffer'). Malonyl-CoA was added (100 μM) as a water-soluble alternative substrate for the acyl-CoA hydrolase. No bromopalmitoyl-CoA inhibition was observed if alternative substrate was not provided. We had previously determined that 20 μM-palmitoyl-CoA provided results equivalent to those with 100 μM-malonyl-CoA, and that malonyl-CoA alone had no effect. At the end of the incubation period, mitochondria were diluted approx. 5-fold with the same buffer containing 0.05% bovine serum albumin and pelleted by centrifugation. The pellet was resuspended and centrifuged in isolation buffer without albumin and then finally resuspended in the isolation buffer for kinetic determinations. In another set of experiments, mitochondria were incubated as above with 50 μM-bromopalmitoyl-L-carnitine with or without 5 mM-CoA. The same incubation and isolation protocol was employed as described above.

Protein concentration was determined by the biuret procedure (Gornall *et al.*, 1949), and adjusted to 2 mg/ml for subsequent CPT determination of intact mitochondria and inverted vesicles. Protein concentration of purified CPT was determined as described by Bradford (1976).

Purification of CPT and antibody generation

Details of the purification of rat liver mitochondrial CPT have been described previously (P. S. Brady & L. J. Brady, 1986). We have noted since those studies that commercial bulk Triton X-100 used in the initial stages of CPT purification may somewhat inhibit activity of the CPT. The inhibition is apparently reversible. However, alternative detergents are available, which do not present this problem. These include Tween 20 (used in the present studies), octyl glucoside and $C_{12}E_8$ (Calbiochem). Antibody to purified CPT was generated in female Balb/c mice as ascites fluid as described by Tung (1983). The antibody was used as defibrinated ascites fluid. No reactivity to rat liver or pigeon breast CAT was noted. Western-blot analysis of the polyclonal antibody to rat liver CPT in total solubilized mitochondria (using a protein-A-linked horseradish peroxidase system for detection; Bio-Rad, Richmond, CA, U.S.A.) yielded a single reactive band which corresponded to the mobility of the purified CPT. Ascites fluid generated to commercial pigeon breast CAT served as control.

Liposomes

This procedure has been described by Noel *et al.* (1985). In brief, liposomes were prepared by sonicating 0.25 mg of cardiolipin or phosphatidylcholine/ml of buffer (20 mM-potassium phosphate/0.5 mM-dithiothreitol/0.5% Tween 20, pH 7.5) to clarity. Purified CPT (7.5 μg/ml) was added and mixed. Detergent was removed with Extractigel D (Pierce). The supernatant was used after centrifugation for 5 min at 12000 *g*. Antibody precipitations were as described previously. Sensitivity to bromopalmitoyl-CoA was assessed as described below.

Assay methods

CPT activity in intact mitochondria and inverted vesicles was measured in the direction of palmitoylcarnitine formation with L-[^{14}C-*Me*]carnitine as substrate (forward reaction). The CPT assay has been described in detail previously (Brady *et al.*, 1985). Palmitoyl-CoA was varied over the range 10–80 μM, and bromopalmitoyl-CoA was used at final concentrations of 0–10 μM and bromopalmitoylcarnitine at 0–50 μM.

The CPT activity in the reverse direction ([*Me*-^{14}C]carnitine release from palmitoyl-L-[*Me*-^{14}C]carnitine) was measured by a kinetic modification of the assay II of Hoppel & Tomec (1972). The final assay volume of 0.2 ml contained 80 mM-KCl, 50 mM-Mops, 1 mg of defatted dialysed bovine serum albumin/ml, 5 mM-CoA, and 60 μM-, 40 μM-, 30 μM- or 20 μM-palmitoyl-L-carnitine (containing palmitoyl-L-[*Me*-^{14}C]carnitine), pH 7.0. Bromopalmitoyl-L-carnitine was added to give final concentrations of 0, 5, 10, 25 and 50 μM. The reaction was initiated by the addition of enzyme protein (5 μg) after 5 min preincubation at 37 °C. The reaction was terminated after 3 min by the addition of 0.2 ml of 6% $HClO_4$.

The reaction was linear to 10 μg of protein for 5 min. The samples were cooled on ice for 30 min, centrifuged at 12000 *g* for 5 min in an Eppendorf Microfuge, and a sample of the supernatant, containing the liberated carnitine, was counted for radioactivity (Hoppel & Tomec, 1972). Controls were run to correct for unprecipitated palmitoyl-[*Me*-^{14}C]carnitine. Where purified CPT was used, the spectrophotometric method of Bieber *et al.* (1972) was used for CPT assay. Dimethyl sulphoxide (Me_2SO) was added at 20% (v/v) where used. Initial dose/response data showed that Me_2SO addition up to 30% increased CPT activity 2–3-fold, with activity declining above 30%.

Sensitivity of CPT to inhibition by bromoacyl derivatives was assessed by plotting the reciprocal velocity versus inhibitor concentration by using two or more palmitate-derivative concentrations (Dixon plot; Segel, 1975). Lines were fitted by linear regression. The projection of the intersection of the lines to the ordinate was taken as $-K_i$. However, the CPT assays with intact and inverted mitochondria contained albumin, hence the K_i is only apparent and is reported as I_{50}. This convention is maintained for the purified CPT to reflect the uncertainty of the exact acyl-derivative concentration, since these species may form micelles, making the exact concentration uncertain.

Statistics

Data were analysed by analysis of variance for a factorial design with the potential to accommodate missing data (Federer & Zelen, 1966), or for completely randomized or randomized block designs (Steel & Torrie, 1960). The S.E.M. was derived from the mean-square error of the analysis of variance. Linear regression analysis was determined by standard least-squares techniques. In all cases where significant ($P < 0.05$) linearity was found, fit was compared with quadratic fit for improvement (Steel & Torrie, 1960). In no case was a significant improvement in fit observed by the higher-order equation.

Materials

Palmitoyl-CoA and bromopalmitoyl-CoA were synthesized as described by Seubert (1960), by using commercially available palmitoyl chloride (Sigma) or bromopalmitoyl chloride synthesized by the reaction of bromopalmitic acid with oxalyl chloride as described by Kiorpes *et al.* (1984) for the generation of tetradecylglycidyl chloride. L-Carnitine was given by Sigma-Tau, Rome, Italy. L-[*Me*-^{14}C]Carnitine was synthesized by using [^{14}C]methyl iodide (Dupont–New England Nuclear), as described by Ingalls *et al.* (1982). Palmitoyl-L-[*Me*-^{14}C]carnitine was synthesized from L-[*Me*-^{14}C]carnitine and palmitoyl chloride as described by Brendel & Bressler (1967), except that carnitine perchlorate was prepared as described by Chase & Tubbs (1972). Bromopalmitoylcarnitine was synthesized by the method of Brendel & Bressler (1967) and isolated as described by Chase & Tubbs (1972). Cytochrome *c*–Sepharose was synthesized by using bovine cytochrome *c* and CNBr-activated Sepharose (Pharmacia) as described by Godinot & Gautheron (1979). Malonyl-CoA was purchased from P-L Biochemicals, Milwaukee, WI, U.S.A. Bovine serum albumin (type V) was defatted (Chen, 1967) and dialysed (Hanson & Ballard, 1968). Me_2SO was purchased from J. T. Baker. DEAE-Toyopearl (DEAE-Fractogel) was purchased from American Scientific Products. All other chemicals were reagent grade or better.

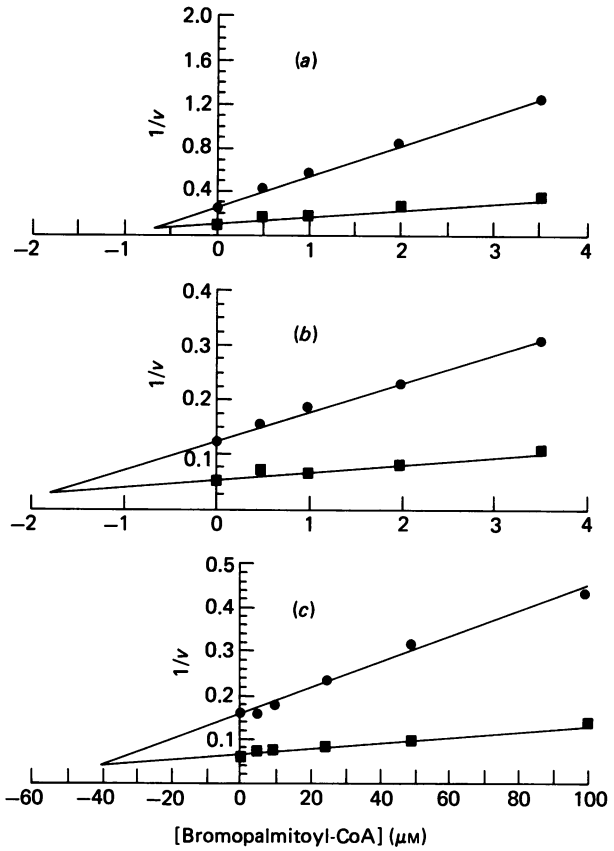

Fig. 1. Representative plots of CPT sensitivity to bromopalmitoyl-CoA inhibition (Dixon plots)

(*a*) CPT activity in intact mitochondria was assayed in the presence of 0–3.5 μM-bromopalmitoyl-CoA and (■) 80 μM- or (●) 10 μM-palmitoyl-CoA. Details of the assay are presented in the Experimental section. The unit of v is nmol/min per mg of mitochondrial protein. (*b*) CPT activity of inverted mitochondria was assayed as in (*a*). (*c*) Purified hepatic CPT activity was assayed spectrophotometrically as described in the Experimental section. The particular plot represents a determination in cardiolipin liposomes, but is generally representative of plots for phosphatidylcholine liposomes or in Me_2SO. Here the unit of v is μmol/min per mg of CPT protein.

RESULTS AND DISCUSSION

Preincubation of intact mitochondria with bromopalmitoyl-CoA and bromopalmitoylcarnitine

Initial studies *in situ* confirmed the results of others that CPT-A was inhibited by bromopalmitoyl-CoA ($I_{50} = 0.63 \pm 0.08$ μM; $n = 6$; Fig. 1*a*) and added new data that CPT-B activity (activity of inverted vesicles) was also inhibited ($I_{50} = 2.44 \pm 0.86$ μM; $n = 4$; Fig. 1*b*). Bromopalmitoylcarnitine did not inhibit the forward reaction in intact mitochondria or in inverted submitochondrial vesicles when competing with either palmitoyl-CoA or L-carnitine. In the reverse reaction, bromopalmitoylcarnitine was competitive with palmitoylcarnitine ($I_{50} = 20.7 \pm 7.0$ μM) in intact mitochondria,

Table 1. Effect of preincubation of intact mitochondria with 2-bromopalmitoyl-CoA

Intact mitochondria were prepared as described in the Experimental section, and divided among three tubes: one received isolation buffer only (control), the second received 50 μM-bromopalmitoyl-CoA + 100 μM malonyl-CoA, and the third received 50 μM-bromopalmitoyl-CoA + 100 μM-malonyl-CoA + 5 mM-L-carnitine. Mitochondria were incubated for 5 min at 25 °C after these additions, and washed once with isolation buffer containing 0.05% albumin and then twice with isolation buffer without albumin. The mitochondria were resuspended in isolation buffer at 2 mg of protein/ml. Kinetic determinations were made on intact mitochondria and inverted submitochondrial vesicles prepared from the mitochondria. Data were analysed by analysis of variance by using a randomized block design. The S.E.M. is derived from the mean-square error of the analysis of variance; *indicates a significant effect of treatment ($P < 0.01$).

	Intact mitochondria				Inverted vesicles			
		Bromopalmitoyl-CoA treated				Bromopalmitoyl-CoA treated		
	Control	−Carnitine	+Carnitine	S.E.M.	Control	−Carnitine	+Carnitine	S.E.M.
$V_{max.}$ (nmol/min per mg)	12.0	6.9	5.2	0.5*	43.5	41.4	30.5	1.5*
$K_{0.5}$ for palmitoyl-CoA (μM)	26.9	15.8	11.8	1.2*	40.9	43.6	27.5	3.8*
K_m for carnitine (mM)	0.16	0.13	0.12	0.02	0.24	0.24	0.15	0.04

Table 2. Effect of preincubation of intact mitochondria with bromopalmitoylcarnitine on CPT kinetics

The conditions of the experiments were as described for Table 1, except that the substrates used during the preincubation were: (1) none (control); (2) 50 μM-bromopalmitoylcarnitine; and (3) 50 μM-bromopalmitoylcarnitine + 5 mM-CoA. Subsequent treatment and analysis of data were described in Table 1.

	Intact mitochondria				Inverted vesicles			
		Bromopalmitoylcarnitine				Bromopalmitoylcarnitine		
	Control	−CoA	+CoA	S.E.M.	Control	−CoA	+CoA	S.E.M.
$V_{max.}$ (nmol/min per mg)	19.1	16.7	13.7	1.3	57.1	35.3	33.3	5.2*
$K_{0.5}$ for palmitoyl-CoA (μM)	28.2	22.0	17.9	2.7	45.5	28.4	38.4	6.8
K_m for carnitine (mM)	0.19	0.22	0.17	0.04	0.28	0.18	0.22	0.03

with no measurable effect on the inverted vesicles. We then turned to the preincubation studies to assess further the relationship of CPT-A and -B. Table 1 presents the results of studies where bromopalmitoyl-CoA was preincubated with intact mitochondria before use of the intact mitochondria directly or to prepare inverted vesicles for kinetic studies. Bromopalmitoyl-CoA addition to the preincubation medium depressed the $V_{max.}$ and $K_{0.5}$ for palmitoyl-CoA of CPT-A by 50%, with no effect on the K_m for carnitine. The decrease in CPT activity in the absence of carnitine may reflect an effect of carnitine efflux from the intact mitochondria. Grantham & Zammit (1986) have previously noted inhibition without added carnitine. They also inhibited CPT-A by 80%, in contrast with the 50% that we report, which probably reflects differences in the incubation and assay conditions. The $V_{max.}$ and $K_{0.5}$ for palmitoyl-CoA of CPT-B activity were depressed by 25%, but only if carnitine was present in the preincubation medium. In the absence of carnitine, the $V_{max.}$ of the inverted vesicles derived from intact mitochondria exposed to bromopalmitoyl-CoA was similar to that of vesicles untreated with bromopalmitoyl-CoA. Obviously, the inverted vesicles have no store of carnitine to support efflux. These data suggest that direct exposure of CPT-A and CPT-B activity to bromopalmitoyl-CoA or bromopalmitoylcarnitine is necessary for loss of activity. In fact, Grantham & Zammit (1986) have proposed that bromopalmitoyl-CoA and carnitine incubated together with intact mitochondria lead to CPT-A inhibition by the self-catalysed formation of the *S*-carboxypalmitoyl-CoA ester of carnitine at the active site, and Saggerson & Carpenter (1986) have presented data in support of this mechanism. This mechanism is the same as that proposed by Chase & Tubbs (1969) for the inhibition of purified pigeon breast-muscle CAT by formation of the *S*-carboxymethyl-CoA carnitine ester. Our experiments to test this hypothesis with purified rat liver CPT are described below.

CPT kinetic parameters were estimated in intact mitochondria and inverted vesicles after exposure of the intact mitochondria to 50 μM-bromopalmitoylcarnitine and subsequent washing, as done for the bromopalmitoyl-CoA experiments (Table 2). There was no effect of bromopalmitoylcarnitine at the concentrations used on $V_{max.}$ or $K_{0.5}$ of CPT in intact mitochondria after exposure and washing, regardless of whether or not CoA was provided in the incubation. When inverted vesicles were prepared from these mitochondria, the $V_{max.}$ of CPT was depressed, with no change in the $K_{0.5}$. The data taken together suggest that, under the conditions employed, CPT-A did not form the *S*-carboxypalmitoyl-

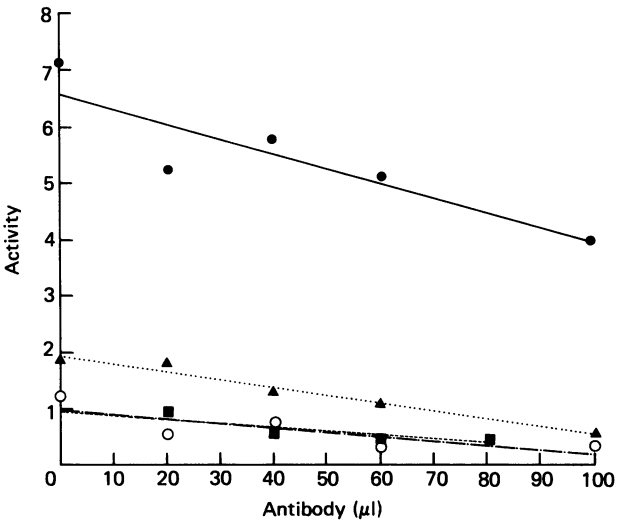

Fig. 2. Antibody interaction with various mitochondrial CPT preparations

Various preparations of mitochondrial CPT were prepared and incubated with crude anti-CPT antibody (defibrinated mouse ascites fluid) for 1 h at room temperature, followed by incubation for 20 min with protein A. Antibody–antigen complex was pelleted by centrifugation, and CPT activity of the supernatant was assayed spectrophotometrically. Activity is expressed as total units (nmol/min). Samples were: ●, purified CPT; ○, Triton X-100-(0.05%) solubilized mitochondria; ■, supernatant after sonication and centrifugation (100000 *g* for 1 h) of isolated mitochondria; ▲, resuspended pellet from the same procedure. See the Experimental section for specific details.

CoA carnitine ester, or that it was formed and dissociated rapidly. Bromopalmitoylcarnitine was inhibitory to CPT-B whether or not CoA was included in the preincubation. Since there is an intramitochondrial CoA pool, this does not rule out the formation of the *S*-carboxypalmitoyl-CoA carnitine ester as the mechanism of inhibition. Indeed, Chase & Tubbs (1972) postulated that bromopalmitoylcarnitine needs to be converted into bromopalmitoyl-CoA to inhibit CPT and that the CoA is derived from the intramitochondrial pool. The mechanism of formation of *S*-carboxypalmitoyl-CoA carnitine ester would also require the presence of intramitochondrial CoA.

Inhibition of sonicated and detergent-solubilized CPT

We have examined the effects of 2-bromopalmitoyl-CoA on detergent-solubilized and sonicated mitochondria and on the 100000 *g* supernatant and pellet after sonication, as suggested by Hoppel (1982). The latter procedure was suggested to dissociate CPT-A from the inner membrane, providing a means of separating CPT-A and CPT-B activity, although it appears that the sonicated supernatant and pellet are equally sensitive to the antibody as solubilized CPT (Fig. 2). We have succeeded in purifying only a single CPT activity from rat liver mitochondria, and the antibody is raised to this activity. However, the antibody is polyclonal, and it may be argued that the similarities between CPT-A and -B are such that a polyclonal antibody would be expected to cross-react. At the same time, Western blots of total solubilized mitochondria showed only a single band, which corresponded to CPT mobility on SDS/polyacrylamide-gel electrophoresis (P. S. Brady & L. J. Brady, unpublished work). Thus, if the supernatant and pellet represent CPT-A and -B, respectively, as distinct proteins, the two proteins are quite similar.

There was no bromopalmitoyl-CoA inhibition (at 0–20 μM) of CPT in mitochondria that had been solubilized with 0.05% Triton X-100. The bromopalmitoyl-CoA I_{50} for mitochondria which were sonicated in hypo-osmotic phosphate/EDTA was 16.4 ± 1.4 μM (mean ± S.E.M.; $n = 3$), for the sonicated 100000 *g* supernatant, 10.2 ± 1.5 μM ($n = 3$), and for the resuspended sonicated 100000 *g* pellet, 10.2 ± 4.7 μM ($n = 3$). These data, when compared with the control I_{50} of 0.63 μM, indicate that these procedures severely decreased the sensitivity of the enzyme to bromopalmitoyl-CoA. Total disruption of the membrane and/or of the enzyme's conformation with Triton abolished inhibition by bromopalmitoyl-CoA at concentrations of 0–20 μM. Membrane structure and/or CPT folding or membrane association may be important factors in the sensitivity, as noted below.

In another series of experiments, mitochondria were incubated with bromopalmitoyl-CoA + [^{14}C]carnitine, solubilized with Triton, and CPT activity was precipitated with antibody or the solubilized preparation was passed over DEAE-Toyopearl as for the purification procedure. Sonicated supernatant and pellet were similarly treated. In each case, the label and the CPT activity were dissociated (results not shown).

Inhibition of purified CPT

Purified hepatic CPT was inhibited by higher concentrations of bromopalmitoyl-CoA ($I_{50} = 353$ μM). In the same spectrophotometric assay, addition of 20% Me_2SO increased sensitivity to bromopalmitoyl-CoA ($I_{50} = 67$ μM). Me_2SO at this concentration decreases water activity (order). It would appear that this process is desirable for access of the acyl-CoA to the active site. This may result from alteration of the CPT conformation or from the exclusion of water from the active site, or both. Indeed, estimated $V_{max.}$ increased from 22 to 38 units/mg of protein, while the palmitoyl-CoA $K_{0.5}$ decreased from 45 μM to 30 μM. With regard to the effect of Me_2SO on inhibition of purified CPT by malonyl-CoA and by tetradecylglycidyl-CoA, Me_2SO at 20% had no effect on malonyl-CoA sensitivity (no inhibition of purified CPT by malonyl-CoA is observed in any case). However, the I_{50} for tetradecylglycidyl-CoA decreased from 65 to 9.5 μM in the presence of 20% Me_2SO. Time-course studies indicated that maximum inhibition of purified CPT by bromopalmitoyl-CoA was attained within the shortest interval measured (5 s). Thus, if an inhibitory intermediate is formed, its formation is very rapid. Further, where purified CPT was preincubated with bromopalmitoyl-CoA, reaction rates with various concentrations of palmitoyl-CoA and L-carnitine were linear, supporting rapid dissociation of the inhibitor. In no case was there evidence that bromopalmitoyl-CoA was other than a competitive inhibitor with the purified CPT. This is in contrast with the data presented for intact mitochondria and inverted vesicles (Table 1), which seem to support a less freely dissociated inhibitor. The differences between the purified system and that *in situ* are

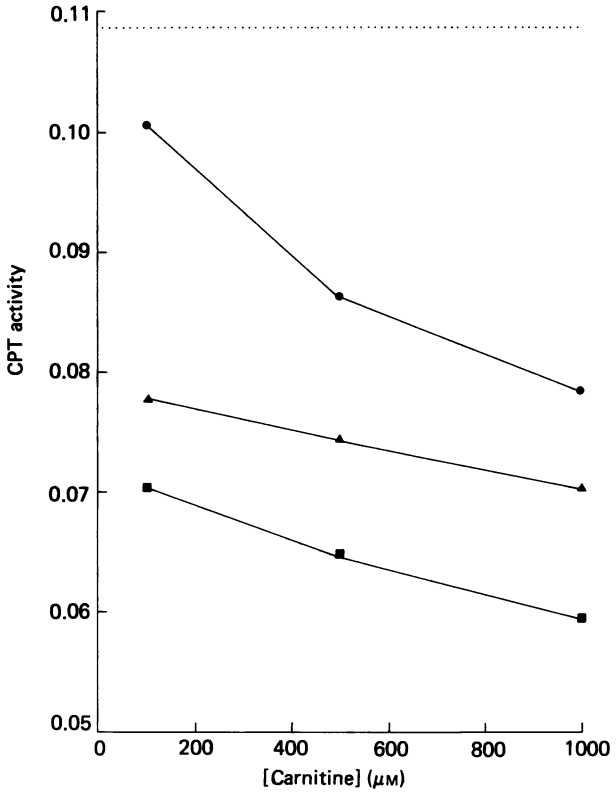

Fig. 3. Effect of various concentrations of L-carnitine and bromopalmitoyl-CoA on CPT activity

CPT activity was determined by using purified CPT exposed to various concentrations of L-carnitine and bromopalmitoyl-CoA (●, 25 μM; ▲, 50 μM; ■, 100 μM) in the standard spectrophotometric assay (see the Experimental section). The reaction was initiated by addition of the enzyme, and activity (as ΔA_{412}/min per 20 μl of a 1:5 dilution of CPT) was determined immediately. The dotted line represents the activity in the absence of bromopalmitoyl-CoA.

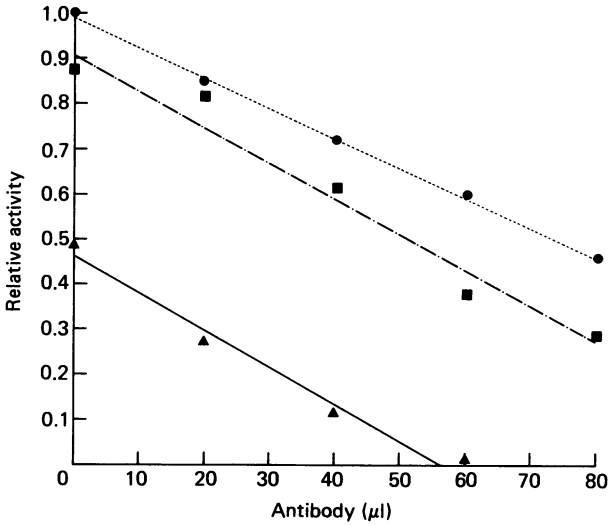

Fig. 4. Antibody precipitation of CPT activity in liposomes

CPT was inserted into cardiolipin (●) or phosphatidylcholine (■) liposomes as described in the Experimental section. The precipitation of the CPT activity of these preparations was compared with that of intact mitochondria after removal of the outer membrane with phospholipase C (▲) as described by Brosnan *et al.* (1973). The antibody reaction was as described in Fig. 2.

of interest, and would suggest that factors other than the simple interaction of bromopalmitoyl-CoA with the CPT catalytic site are involved. Such factors include possible sequestration of bromopalmitoyl-CoA or bromopalmitoyl-CoA carnitine ester in the membrane or within the particle, direct modification of the membrane, or interaction of bromopalmitoyl-CoA and/or the carnitine ester with the malonyl-CoA site or other regulatory sites.

Studies of the effect of pH on bromopalmitoyl-CoA sensitivity of purified CPT (using the radioactive 'forward reaction') suggested that bromopalmitoyl-CoA inhibition as a percentage of control was greater at pH 6–7 than at pH 8.0 (results not shown). If the mechanism proposed by Chase & Tubbs (1969) applies, the data indicate increased disruption of the *S*-carboxypalmitoyl-CoA carnitine ester at pH 8.0.

Preincubation of purified CPT with bromopalmitoyl-CoA + carnitine

Experiments with purified hepatic CPT support the hypothesis of the formation of the *S*-carboxypalmitoyl-CoA carnitine ester, as proposed by Chase & Tubbs (1969) for CAT and by Grantham & Zammit (1986) for CPT, but not for an irreversible complexing of inhibitor to CPT. When various concentrations of bromopalmitoyl-CoA and carnitine were preincubated with purified CPT for 1 min, the spectrophotometric assay of CPT in the forward direction indicated increased inhibition of CPT activity with increasing preincubation concentrations of carnitine and bromopalmitoyl-CoA (Fig. 3). At any given bromopalmitoyl-CoA concentration, inhibition increased with increasing [carnitine] in the preincubation. Incubation of purified CPT + 50 μM-bromopalmitoyl-CoA + 2 μM-L-[*Me*-^{14}C]carnitine for 5 min at room temperature in the Mops/sucrose/mannitol buffer led to ^{14}C accumulation associated with the CPT (trichloroacetic acid precipitated) as compared with a control sample incubated with palmitoyl-CoA rather than bromopalmitoyl-CoA. The difference spectrum (24 μg of CPT + 50 μM-bromopalmitoyl-CoA + 2 μM-L-[*Me*-^{14}C]carnitine versus 24 μg of CPT + 50 μM-palmitoyl-CoA + 2 μM-L-[*Me*-^{14}C]carnitine) was similar to that observed by Chase & Tubbs (1969) for CAT (results not shown). Inhibition was instantaneous, as was observed also for CAT (Chase & Tubbs, 1969). Unlike with CAT, the inhibition was reversed by less than 24 h of dialysis. Also, incubation of CPT with bromopalmitoyl-CoA + [^{14}C]carnitine followed by antibody precipitation of the CPT failed to pellet significant radioactivity, whereas over 90% of CPT activity was removed from the suspension. Tetradecylglycidyl-CoA (100 μM) and malonyl-CoA (100 μM) did not alter inhibition by bromopalmitoyl-CoA when these inhibitors were added with bromopalmitoyl-CoA and carnitine, followed by immediate measurement of CPT activity. This suggests that the site(s) of interaction of bromopalmitoyl-CoA and of malonyl-CoA and tetradecylglycidyl-CoA may differ.

CPT re-insertion into liposomes

Insertion of purified CPT into liposomes increased sensitivity to bromopalmitoyl-CoA ($I_{50} = 12\ \mu M$ for phosphatidylcholine liposomes and 40 μM for cardiolipin liposomes; Fig. 1*c*). Thus the CPT in liposomes was slightly more sensitive to inhibition than was CPT measured in Me_2SO. Again, this points to the importance of the specific membrane environment to the expression of CPT characteristics. The inner surface of the inner membrane is enriched in cardiolipin (Daum, 1985). This may be important for expression of activity and kinetic constants. However, little effect of cardiolipin was seen on bromopalmitoyl-CoA sensitivity. This may have been expected, since only minor differences in CPT sensitivity to bromopalmitoyl-CoA between intact mitochondria and inverted vesicles were observed *in situ*. CPT activity was equally accessible to antibody in either vesicle preparation (Fig. 4), although cardiolipin liposomes showed higher activity than the phosphatidylcholine liposomes. We (L. J. Brady & P. S. Brady, 1986) and others (Pande *et al.*, 1986) have previously noted that cardiolipin appears to stimulate the CPT forward reaction.

In conclusion, the formation of the *S*-carboxypalmitoyl-CoA carnitine ester appears to occur for the CPT reaction. However, this intermediate is freely dissociated from purified CPT or on solubilization of intact mitochondria. Still, we believe that the use of bromopalmitoyl-CoA and anti-CPT antibody has helped to approach two major questions relative to CPT. Our first question was whether there are, indeed, two CPT proteins. Although two distinct proteins cannot be ruled out, the CPT characteristics of intact mitochondria and inverted vesicles were not grossly different in their response to bromopalmitoyl-CoA. Also, the preparations were antigenically similar, at least to polyclonal antibody. However, sensitivity of CPT to bromopalmitoyl-CoA appears to be strongly dependent on the relationship of the CPT to the membrane. Differences between CPT-A and CPT-B activities may, once again, be more a question of environmental influences than discrete structural differences in the protein or proteins. Our second question was whether malonyl-CoA and tetradecylglycidyl-CoA interact with the same site as bromopalmitoyl-CoA. Malonyl-CoA appears to exhibit a form of interaction distinctly different from that of bromopalmitoyl-CoA. Evidence for this comes from the failure of malonyl-CoA to interfere with bromopalmitoyl-CoA inhibition of CPT, as well as the total lack of response of CPT to malonyl-CoA in the presence of Me_2SO. Tetradecylglycidyl-CoA poses a more difficult problem. At the concentrations used, it did not appear to protect purified CPT against bromopalmitoyl-CoA inhibition. Still, Me_2SO resulted in a significant increase in CPT sensitivity to tetradecylglycidyl-CoA. Although further study is required, the simplest explanation is that tetradecylglycidyl-CoA interacts both with the malonyl-CoA site and with the same site (albeit with low affinity) as bromopalmitoyl-CoA. In all probability, the site of interaction of bromopalmitoyl-CoA is the CPT catalytic site.

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