Reconstitution of purified, active and malonyl-CoA-sensitive rat liver carnitine palmitoyltransferase I: relationship between membrane environment and malonyl-CoA sensitivity

J. Denis McGARRY*† and Nicholas F. BROWN*¹

*Department of Internal Medicine, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75235-9135, U.S.A., and

†Department of Biochemistry, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75235-9135, U.S.A.

Carnitine palmitoyltransferase I (CPT I) catalyses the initial step of fatty acid import into the mitochondrial matrix, the site of β -oxidation, and its inhibition by malonyl-CoA is a primary control point for this process. The enzyme exists in at least two isoforms, denoted L-CPT I (liver type) and M-CPT I (skeletal-muscle type), which differ in their kinetic characteristics and tissue distributions. A property apparently unique to L-CPT I is that its sensitivity to malonyl-CoA decreases *in vivo* with fasting or experimentally induced diabetes. The mechanism of this important regulatory effect is unknown and has aroused much interest. CPT I is an integral outer-membrane protein and displays little activity after removal from the membrane by detergents, precluding direct purification of active protein by conventional means. Here we describe the expression of a 6 $\times$ His-tagged rat L-CPT I in *Pichia pastoris* and purification of the detergent-solubilized enzyme in milligram quantities. Reconstitution of the purified product into a liposomal environment yielded a 200–400-fold increase in enzymic activity and restored malonyl-CoA sensitivity. This is the first time that a CPT I protein has been available for study in a form that is both pure and active. Comparison of the kinetic properties of the reconstituted material with those of L-CPT I as it exists in mitochondria prepared from yeast over-expressing the enzyme and in livers from fed or fasted rats permitted novel insight into several aspects of the enzyme's behaviour. The malonyl-CoA response of the liposomal enzyme was found to be greater when the reconstitution procedure was carried out at 22 °C compared with 4 °C ($IC_{50} \approx 11 \mu M$ versus $30 \mu M$, respectively). When the sensitivities of L-CPT I in each of the different environments were compared, they were found to decrease in the following

order: fed liver > fasted liver $\approx$ liposomes prepared at 22 °C $\approx$ *P. pastoris* mitochondria > liposomes prepared at 4 °C. In addition, pre-treatment of L-CPT I liposomes with the membrane-fluidizing reagent benzyl alcohol caused desensitization to the inhibitor. In contrast with the variable response to malonyl-CoA, the liposomal L-CPT I displayed a pH profile and kinetics with regard to the carnitine and acyl-CoA substrates similar to those of the enzyme in fed or fasted liver mitochondria. However, despite a normal sensitivity to malonyl-CoA, L-CPT I in *P. pastoris* mitochondria displayed aberrant behaviour with regard to each of these other parameters. The kinetic data establish several novel points. First, even after stringent purification procedures in the presence of detergent, recombinant L-CPT I could be reconstituted in active, malonyl-CoA sensitive form. Second, the kinetics of the reconstituted, 6 $\times$ His-tagged L-CPT I with regard to substrate and pH responses were similar to what is observed with rat liver mitochondria (whereas in *P. pastoris* mitochondria the enzyme behaved anomalously), confirming that the purified preparation is a suitable model for studying the functional properties of the enzyme. Third, wide variation in the response to the inhibitor, malonyl-CoA, was observed depending only on the enzyme's membrane environment and independent of interaction with other proteins. In particular, the fluidity of the membrane had a direct influence on this parameter. These observations may help to explain the mechanism of the physiological changes in the properties of L-CPT I that occur *in vivo* and are consistent with the current topographical model of the enzyme.

Key words: fatty acid, liposome, mitochondria.

INTRODUCTION

The carnitine palmitoyltransferase (CPT) system consists of three enzymes that operate sequentially to effect the net transport of long-chain fatty acids from the cytosol into the mitochondrial matrix, the site of β -oxidation. The first component, CPT I, an integral protein of the outer mitochondrial membrane, catalyses the transfer of a fatty acyl group from cytosolic acyl-CoA to carnitine. The acylcarnitine formed is then able to enter the mitochondrial matrix by means of a specific carnitine/acylcarnitine carrier protein. A distinct gene product, CPT II, loosely associated with the inner aspect of the inner membrane,

reverses the CPT I reaction, regenerating fatty acyl-CoA and releasing free carnitine. The acetyl-CoA formed may be completely oxidized via the tricarboxylic acid cycle for the direct production of energy or, in the liver, may serve as a substrate for the synthesis of ketone bodies [1].

In addition to catalysing the first step in this transport process, CPT I plays a crucial role in the physiological regulation of fatty acid oxidation. This is mediated through potent inhibition of the enzyme by malonyl-CoA, the product of acetyl-CoA carboxylase and the first committed intermediate in the opposing pathway of fatty acid synthesis *de novo*. Thus in the liver, in the fed state, fatty acid synthesis is activated and malonyl-CoA levels

Abbreviations used: CPT, carnitine palmitoyltransferase; L-CPT I, liver-type CPT I; M-CPT I, skeletal-muscle-type CPT I; Ni-NTA, Ni^{2+} -nitrilotriacetate.

¹ To whom correspondence should be addressed (e-mail nbrown@mednet.swmed.edu).

rise, CPT I is inhibited and futile oxidation of the newly synthesized fatty acids is prevented. Conversely, during a fast, hepatic malonyl-CoA levels fall, such that β -oxidation and ketone-body synthesis can proceed efficiently (see [1] for a review). Although first described for the liver, it now appears that this system operates in non-lipogenic tissues as well. In skeletal muscle and heart, for example, acetyl-CoA carboxylase activity is present and malonyl-CoA levels are seen to rise and fall with changes in physiological state, just as in liver [2–5]. In these tissues, it is thought that malonyl-CoA is synthesized purely as a regulator of CPT I. Because of its central role in whole-body fuel homeostasis, CPT I has aroused interest as a target for pharmacological intervention in conditions of excessive fatty acid oxidation. For example, CPT I inhibitors are considered potentially valuable as hypoglycaemic agents in the long-term management of type-II diabetes or in the short-term reversal of diabetic ketoacidosis [6,7], as well as in the treatment of ischaemic heart disease where excessive oxidation of fatty acids is believed to exacerbate cardiac tissue damage [8–10].

Whereas CPT II, the inner-membrane (and malonyl-CoA-insensitive) form of CPT, is thought to be the same protein in all tissues of the body, CPT I is known to exist in at least two primary isoforms [1,11,12]. These have been designated L-CPT I (liver type) and M-CPT I (skeletal-muscle type) since they appear to be the exclusive isoforms expressed in those organs, although each is found in a variety of other tissues [1]. In addition, based on analysis of mRNA transcripts, it has been suggested that alternatively spliced forms of M-CPT I may be expressed [13–15]. L- and M-CPT I are predicted to be proteins of 773 and 772 amino acid residues, respectively, and are 63% identical at the amino acid level [16]. However, they differ in several important respects. Notably, the K_m for the substrate carnitine is 10–15-fold higher in the case of M-CPT I ($\approx 500 \mu\text{M}$) and, whereas the IC_{50} for malonyl-CoA is $\approx 0.03 \mu\text{M}$ for M-CPT I, it is 100-fold higher for the liver enzyme [1]. Also, despite having almost identical predicted relative molecular masses, the two CPT I isoforms display different mobilities on SDS/PAGE [12].

An intriguing property unique to L-CPT I is that its sensitivity to malonyl-CoA changes with (patho)physiological state. When mitochondria are isolated from the livers of rats that have been fasted or rendered experimentally diabetic, L-CPT I is found to be markedly desensitized to malonyl-CoA when compared with those prepared from a fed animal [17–19]. In the case of fasting, this phenomenon of desensitization to the inhibitor is believed to act in physiological terms to amplify the effect of a decreased malonyl-CoA concentration. However, the mechanism of this change in the molecule's behaviour is unknown and remains controversial.

Each of the CPT cDNAs has now been cloned from rat, human and other species [1] and this has facilitated a number of recombinant expression studies that have provided some insight into which regions and/or amino acid residues within the CPT molecules bestow particular properties. In addition, a membrane topology has been proposed that envisages CPT I as consisting of a small N-terminal domain and a larger C-terminal domain, both lying on the cytosolic aspect of the outer mitochondrial membrane and connected by two membrane spans with a short loop in the inter-membrane space [20–23]. The C-terminal region contains the active site and the determinants for whether the molecule will display L- or M-CPT I-type kinetics with regard to the K_m for carnitine or sensitivity to malonyl-CoA [23]. However, the N-terminal domain is crucial for a normal response to malonyl-CoA [23–27].

A practical problem that has hampered further progress in this area is the fact that CPT I is largely inactivated upon detergent

extraction from the membrane [24,28]. This effect, presumably reflecting the necessity for a membrane environment to maintain the native tertiary structure of the protein, has made it impossible to obtain CPT I in a form that is both pure and active. In the present study we have overcome this obstacle. Rat liver CPT I, expressed at high levels in the yeast *Pichia pastoris* and purified from detergent extracts in milligram quantities, was reconstituted into artificial phospholipid liposomes with concomitant re-establishment of catalytic activity and malonyl-CoA sensitivity. This provides the first opportunity to study the properties of a CPT I isoform in active form in a defined environment free of contaminating proteins. The liposomal CPT I preparation has allowed us to demonstrate a direct effect of membrane environment on the malonyl-CoA sensitivity of the enzyme and has yielded additional insight into the relationship between the enzyme's catalytic and inhibitory sites. The data shed new light on the phenomenon of the desensitization of L-CPT I to its physiological inhibitor.

EXPERIMENTAL

Animals

Male Sprague–Dawley rats (200–250 g) were housed in a room lit from 10:00 h until 22:00 h, and maintained on a high-carbohydrate/low-fat diet [60% sucrose/20% protein/<0.1% fat (w/w)]. Fasted rats were deprived of food from 16:00 h 2 days prior to experiments (i.e. approx. 42 h of fast). Animals were killed at 09:30 h by injection of Nembutal (50 mg/kg) followed by cervical dislocation. Liver mitochondria were prepared at 4 °C, as described previously ([2], method A), and resuspended in 150 mM KCl/5 mM Tris/HCl, pH 7.2.

Expression of rat L-CPT I in *P. pastoris*

Engineering of plasmid pCMV6-rL-CPT I containing the full-length cDNA for rat L-CPT I has been described [11]. With that plasmid as template, the PCR was used to amplify the coding region of the cDNA. The 5' primer oligonucleotide, complementary to the first 21 bases of the open reading frame, contained, in addition, an *Sfi*I restriction site (CCCGA-ATTCGAAATAATGGCAGAGGCTCACCAAGCT). The 3' primer encoded the last 21 bases of the coding region as well as six histidine residues, a stop codon and an *Sfi*I site (CCCGA-ATTCGAATTAATGGTGATGGTGATGGTGCTTTTGAATTGATGGTGAG). The product was ligated into the unique *Sfi*I site of plasmid pPICZA (Invitrogen, Carlsbad, CA, U.S.A.). This placed the rat L-CPT I cDNA under the control of the methanol-inducible AOX1 promoter. The fidelity of the amplification reaction was confirmed by DNA sequencing and the final product is designated pPICZA-rL-CPT I-his6. The plasmid was linearized with restriction enzyme *Pme*I and used to transform *P. pastoris* strain GS115 employing the EasyComp method (Invitrogen) to generate stable chromosomal integrants. Transformed colonies were selected using the Zeocin resistance encoded by the parent plasmid. Eight colonies were chosen for initial study and the highest-expressing strain, GS115-776-rL-1, was used for further experiments. (Growth and preparation of homogenates for characterization of these strains was essentially by a small-scale version of the procedure described below.)

Preparation of *P. pastoris* mitochondria and membrane fractions

A single colony of GS115-776-rL-1 was used to inoculate starter cultures in MGYH [minimal glycerol medium with histidine; 1.34% (w/v) yeast nitrogen base without amino acids/1% (v/v)

glycerol/ 4×10^{-5} % (w/v) biotin/0.004 % (w/v) histidine] which were grown with shaking overnight at 30 °C. Induced cultures were inoculated from these to an initial D_{600} of 1.0 in MMH [minimal methanol medium with histidine; as MGYH, but with 1 % (v/v) methanol and without glycerol] and grown for 24 h.

A mitochondrially enriched fraction was prepared from 400-ml induced cultures as described previously for *Saccharomyces cerevisiae* [29]. Briefly, the cell wall was removed enzymically, the spheroplasts were broken using 20 cycles of a glass homogenizer fitted with a tight pestle and a mitochondrial fraction was isolated by differential centrifugation. The final product was resuspended in 150 mM KCl/5 mM Tris/HCl, pH 7.2. To obtain preparations of total *P. pastoris* membranes, six 400-ml induced cultures were harvested, after which spheroplasts were prepared and homogenized as above in a final volume of ≈ 80 ml of 0.6 M mannitol/10 mM Tris, pH 7.4/1 mM PMSF. This was divided into four equal portions and centrifuged at 100 000 *g* for 30 min at 4 °C. The resulting membrane pellets were frozen in liquid nitrogen and stored at -70 °C.

Purification of recombinant rat L-CPT I

A membrane pellet prepared as above was thawed rapidly, resuspended in 22.5 ml of 500 mM NaCl/50 mM sodium phosphate, pH 8.0/1 mM PMSF/2 mM mercaptoethanol and homogenized. To this was added 2.5 ml of 10 % (v/v) Triton X-100 to give a final detergent concentration of 1 %. Extraction of the membrane proteins was allowed to proceed with gentle stirring for 1 h at 4 °C, followed by centrifugation at 100 000 *g* for 30 min at 4 °C and collection of the supernatant (≈ 25 ml). A 50 % slurry of Ni²⁺-nitrilotriacetate (Ni-NTA)-agarose (2 ml; Qiagen, Chatsworth, CA, U.S.A.) was washed twice with 3 ml of loading buffer (500 mM NaCl/50 mM sodium phosphate, pH 8.0/10 mM imidazole/1 % Triton X-100/2 mM mercaptoethanol) and added to the membrane extract. Imidazole (5 M, 50 μ l) was also added (to give a final concentration of 10 mM) so that the preparation was now essentially in loading buffer. The mixture was again stirred gently at 4 °C for 1 h before centrifugation at 500 *g* for 5 min. The matrix was washed twice with 4 ml of loading buffer and finally resuspended in the same volume. The slurry was transferred to a glass column (1 cm diameter) fitted with a sintered glass frit and the flow rate adjusted to ≈ 0.2 ml/min. The resulting matrix bed (≈ 1 ml) was then washed sequentially with 10 ml of loading buffer/20 ml of 1 M NaCl/50 mM sodium phosphate, pH 8.0/20 mM imidazole/1 % Triton X-100/2 mM mercaptoethanol and 3 ml of 500 mM NaCl/50 mM sodium phosphate, pH 8.0/20 mM imidazole/0.1 % Triton X-100/2 mM mercaptoethanol. Elution was carried out with 2.5 ml of 500 mM NaCl/50 mM sodium phosphate, pH 8.0/250 mM imidazole/0.1 % Triton X-100/2 mM mercaptoethanol. The entire purification was performed in the cold.

Reconstitution of rat L-CPT I

For reconstitution experiments, 2 ml of the Ni-NTA-agarose column eluate was applied to a Centricon 10 microconcentrator (Amicon, Beverly, MA, U.S.A.) and centrifuged at 5000 *g* for 45 min at 4 °C, which reduced the volume to ≈ 450 μ l. The reconstitution procedure was a modification of that of Garlid and co-workers [30]. To prepare the phospholipids, 50 mg of asolectin (from soya bean, primarily phosphatidylcholine) was dissolved in 500 μ l of chloroform in a glass vial, dried under a stream of nitrogen, redissolved in ≈ 1 ml of diethyl ether and once again dried. It was then solubilized in 650 μ l of 20 mM Hepes, pH 7.2, plus 100 μ l of octyl polyoxyethylene, by heating

at 50 °C with frequent mixing. The lipid was cooled to 4 °C before addition of 300 μ l of the concentrated recombinant protein. To prepare the BioBeads SM2 adsorbent (Bio-Rad, Richmond, CA, U.S.A.), 1.5 g was placed in a 14-ml capped plastic tube and washed twice with 3 ml of 20 mM Hepes, pH 7.2. The washing buffer was removed to leave a final volume of ≈ 2.5 ml. The lipid/protein mixture was added and rocked for 60 min at 4 or 22 °C (see the Results section) and the contents were then transferred to a disposable spin column fitted with a paper frit. Proteoliposomes were harvested by centrifugation at 400 *g* for 2 min.

Assay of CPT activity

CPT I was assayed in the direction of acylcarnitine formation using palmitoyl-CoA and carnitine as substrates [12]. Except where noted, final concentrations in the assay were: 500 μ M L-carnitine (including 0.125 μ Ci of L-[¹⁴C]carnitine), 50 μ M palmitoyl-CoA, 4 mM ATP, 4 mM MgCl₂, 0.25 mM glutathione, 4 mM MgCl₂, 40 μ g/ml rotenone, 2 mM KCN, 15 mM KCl, 1 % (w/v) BSA and 105 mM Tris/HCl, pH 7.2. Malonyl-CoA was added as indicated. For pH curves, to provide buffering over a wide range, the assay was as above, but 35 mM each of Tris, Tes and Mes buffers were substituted for the 105 mM Tris. Assays were performed over 4–6 min, during which time reaction rates were linear. In certain experiments, to artificially fluidize the liposomal membrane, a portion of the proteoliposomal preparation was made 40 mM with respect to benzyl alcohol. This and a control sample were then placed at room temperature (≈ 22 °C) for 8 min before assay.

Protein measurement

The BCA (bicinchoninic acid) protein assay reagent (Pierce, Rockford, IL, U.S.A.) was used with BSA as standard. In the case of fractions from the L-CPT I purification and the proteoliposomes, interfering substances were present. To remove these, the following precipitation procedure was carried out. Samples were made up to a volume of 50 μ l with 20 mM Hepes, pH 7.2, in a Microfuge tube, 250 μ l of -20 °C ethanol/ether (3:1, v/v) was added and the tubes were placed at -20 °C for 30 min. They were then centrifuged at 16 000 *g* for 10 min at 4 °C and the supernatant was removed. The pellet was resuspended with 250 μ l of ethanol/ether (1:1, v/v) and the precipitation and centrifugation steps were performed similarly. The process was then repeated with ethanol/ether (1:3, v/v) and finally with ether alone. The protein pellet was allowed to air dry for 5 min before resuspension with 50 μ l of 5 % SDS in 0.1 M NaOH.

RESULTS

Plasmid pPICZA-rL-CPT I-his6 was designed for expression of recombinant rat L-CPT I in the yeast *P. pastoris*. To facilitate rapid purification of the recombinant product, bases encoding a hexahistidine tag (6 $\times$ His) were introduced at the 3' end of the open reading frame. As a result, the expressed enzyme would possess an additional six histidine residues at its C-terminus, permitting purification over a metal-chelate affinity matrix such as Ni-NTA-agarose. After transformation of *P. pastoris* strain GS115 with plasmid pPICZA-rL-CPT I-his6, eight colonies were analysed for the presence of CPT activity. Small cultures of each were induced with methanol as sole carbon source and total homogenates prepared. All displayed significant catalytic activity, whereas a control strain transformed with the vector alone had none. The highest-expressing strain, GS115-776-rL-1, was

Table 1 Reconstitution of rat L-CPT I

Samples were prepared and assayed as described under the Experimental section. All data are the means $\pm$ S.D. of the number of independent determinations indicated in parentheses. Activity in the presence of 100 μ M malonyl-CoA is given as a percentage of the value in the absence of inhibitor. n.d., not determined.

	CPT activity (nmol/min per mg of protein)	+ 100 μ M malonyl-CoA (%)
<i>P. pastoris</i> membranes	14.9 $\pm$ 4.2 (6)	30.7 $\pm$ 7.3 (6)
Triton X-100 supernatant	0.23 $\pm$ 0.06 (3)	n.d.
Triton X-100 pellet	0.117 $\pm$ 0.08 (3)	n.d.
Ni-NTA-agarose eluate	1.49 $\pm$ 0.5 (5)	114.7 $\pm$ 21.2 (5)
Concentrated eluate	1.08 $\pm$ 0.7 (5)	131.0 $\pm$ 35.0 (4)
Proteoliposomes (4 °C)	245 $\pm$ 124 (5)	37.8 $\pm$ 4.3 (4)
Proteoliposomes (22 °C)	473 $\pm$ 134 (3)	22.7 $\pm$ 3.2 (3)

Figure 1 Purification of recombinant rat L-CPT I from *P. pastoris*

SDS/PAGE analysis of fractions from a typical purification. The same proportion of each fraction was used (0.05%), except for the right-hand proteoliposome lane where 5-fold more was loaded. Triton X-100 extract, total membrane proteins solubilized by the detergent; unbound, material remaining in solution after incubation with Ni-NTA agarose; washes, combined column washes; eluate, material eluted from the column by 250 mM imidazole; matrix, material retained by the column; proteoliposomes, material after reconstitution.

chosen for further analysis. For purification of the recombinant product, large cultures were induced and total membrane fractions prepared (see the Experimental section). Membrane pellets corresponding to 600 ml of original culture were solubilized with 1% Triton X-100 and the insoluble material was sedimented. Western blots indicated an almost quantitative extraction of recombinant L-CPT I (results not shown). CPT I activity, both in the supernatant and pellet fractions, was largely abolished by the Triton X-100 extraction procedure (Table 1), consistent with many previous observations that L-CPT I is largely inactivated upon removal from its membrane environment. Incubation of the solubilized enzyme with Ni-NTA-agarose resulted in specific binding of the 6 $\times$ His-labelled protein. Figure 1 shows SDS/PAGE analysis of a typical purification. Comparison of lane 1 (total Triton X-100 extract) with lane 2 (unbound material) clearly shows that the band at $\approx$ 88 kDa corresponding to L-CPT I was removed by incubation with the matrix. The strength of binding permitted washing of the column under severe conditions (1 M NaCl/1% Triton X-100) and the eluted product was brought to a high degree of purity in a single

step (lane 4). The yield of purified recombinant L-CPT I was $\approx$ 2 mg/l of culture.

As shown in Table 1, despite its purified state, the eluted material retained a much lower specific activity than the original preparation of total yeast membranes. Furthermore, in contrast with the starting material, the low activity detected in the column eluate was not inhibited by malonyl-CoA. Removal of detergent and combination of the purified protein with phospholipid to generate proteoliposomes was carried out at either 4 or 22 °C. Either procedure resulted in a massive (200–400-fold) enhancement of specific activity and, additionally, resurrection of malonyl-CoA sensitivity similar to what was observed in the initial membrane preparation. (Differences shown in Table 1 between the specific activities of proteoliposomes reconstituted at different temperatures are not significant and, indeed, in two experiments in which both variations were carried out simultaneously with portions of the same purified enzyme preparation, no differences in specific activity were observed.) Interestingly, the enzyme reconstituted at 22 °C appeared to display a slightly greater sensitivity to inhibition by malonyl-CoA (Table 1). To ensure that the increase in specific activity upon reconstitution was not simply due to the removal of the detergent (rather than incorporation into phospholipid vesicles) an identical experiment was performed with the omission of the lipid component. The resulting material was totally devoid of activity and, furthermore, a visible precipitate was formed (results not shown). When account is made for sample volumes and losses of L-CPT I protein during the extraction, binding, elution, concentration and reconstitution stages, the final yield of CPT activity was $\approx$ 15% of that in the starting material.

To validate that the L-CPT I had indeed been incorporated successfully into liposomes, we examined the sedimentation properties of the reconstituted product. Centrifugation of the L-CPT I proteoliposomes at 2000 *g* for 2 min resulted in almost complete retention of activity in the supernatant fraction (96.8 $\pm$ 4.4% recovered in the supernatant and 3.1 $\pm$ 0.9% in the pellet, relative to starting activity of 100%). In contrast, when the proteoliposomes were subjected to high-speed centrifugation (150000 *g* for 1 h), the largely cleared supernatant contained very little activity (3.0 $\pm$ 1.7%), most of which was recovered in the liposomal pellet (63.4 $\pm$ 8.6%). Brief sonication of the proteoliposomes caused no change in either the activity or malonyl-CoA sensitivity of the reconstituted material (results not shown). This would suggest that (i) the majority of the enzyme was present in the correct orientation, i.e. N- and C-termini outside, or (ii) if a significant fraction of the liposomal L-CPT I had the opposite orientation, the enzyme present on the inside aspect of the phospholipid bilayer had ready access to both substrates.

Figure 2 Effect of malonyl-CoA on L-CPT I

Values are given as percentages of the activity in the absence of inhibitor [means $\pm$ S.D. for three (or six, 4 °C liposomes only) independent preparations]. Absolute values at 0 μ M malonyl-CoA were: fed liver mitochondria, 8.16 ± 1.83 ; fasted liver mitochondria, 20.9 ± 8.7 ; *P. pastoris* mitochondria, 4.39 ± 0.72 ; proteoliposomes prepared at 4 °C, 245 ± 124 ; proteoliposomes prepared at 22 °C, 473 ± 134 nmol/min per mg of protein. Note that the abscissa is on a logarithmic scale.

In addition to a direct kinetic analysis of the L-CPT I reconstituted at 4 or 22 °C, we performed parallel experiments with a mitochondrial fraction prepared from the *P. pastoris* strain expressing the recombinant enzyme and also with mitochondria from the livers of fed and fasted rats. Figure 2 shows the effect of malonyl-CoA on each of these preparations. The IC_{50} for L-CPT I in fed liver mitochondria was $\approx 3 \mu$ M. As expected, fasted liver mitochondria were somewhat less sensitive, with an IC_{50} of $\approx 8 \mu$ M. L-CPT I *in situ* in *P. pastoris* mitochondria was slightly less sensitive than that of the fasted liver. The temperature at which reconstitution of the purified L-CPT I was performed had a marked effect on the malonyl-CoA sensitivity of the proteoliposomal enzyme. L-CPT I reconstituted at 22 °C had an IC_{50} of $\approx 11 \mu$ M but this value increased to $\approx 31 \mu$ M with material reconstituted at 4 °C. The kinetic data are summarized in Table 2.

The K_m values for carnitine of the fed and fasted rat liver mitochondrial L-CPT I were identical, at about 60μ M (Table 2). Relative to the liver mitochondria, the carnitine curve for the liposomal enzyme was slightly left-shifted with a calculated K_m of

Figure 3 Effect of pH on L-CPT I activity

(A) Activities are normalized as percentages of those values at pH 7.2 in the absence of malonyl-CoA; solid lines, activity without malonyl-CoA; dashed lines, activity in the presence of 5 μ M malonyl-CoA. (B) The activity in the presence of 5 μ M malonyl-CoA is presented as percentages of those values in its absence. All data are means $\pm$ S.D. for three experiments with independent preparations.

$\approx 40 \mu$ M (regardless of the reconstitution temperature). However, L-CPT I in *P. pastoris* mitochondria showed a substantially greater divergence, with a K_m of only $\approx 18 \mu$ M. As regards the response to an increasing concentration of palmitoyl-CoA,

Table 2 Kinetic characteristics of rat L-CPT I in different preparations

All data are means $\pm$ S.D. of three or six (*) independent preparations. IC_{50} , concentration of inhibitor required for 50% inhibition of enzyme activity. K_m (carnitine) data were derived from Eadie-Hofstee linearization plots of the carnitine curve data. The response to palmitoyl-CoA is presented as the concentration required for 50% of the activity at 300 μ M ($S_{300/2}$).

	IC_{50} (malonyl-CoA; μ M)	K_m (carnitine; μ M)	$S_{300/2}$ (palmitoyl-CoA; μ M)
Fed rat liver mitochondria	3.0 ± 0.2	61.9 ± 4.7	25.3 ± 4.0
Fasted rat liver mitochondria	7.8 ± 2.3	62.7 ± 1.2	34.7 ± 5.8
<i>P. pastoris</i> mitochondria	12.0 ± 2.6	17.9 ± 1.2	94.0 ± 2.0
Rat L-CPT I proteoliposomes (4 °C)	$31.5 \pm 6.0^*$	37.3 ± 6.0	34.0 ± 2.6
Rat L-CPT I proteoliposomes (22 °C)	11.4 ± 2.0	38.7 ± 0.4	28.2 ± 4.1

Figure 4 Effect of benzyl alcohol on malonyl-CoA sensitivity of reconstituted L-CPT I

Values are given as percentages of the activity in the absence of inhibitor (means $\pm$ S.D. for three independent preparations). Panels show L-CPT I liposomes reconstituted at (A) 22 °C or (B) 4 °C. Data are presented for samples in the absence (solid lines) or presence (dashed lines) of 40 mM benzyl alcohol (see the Experimental section). Note that the abscissa is on a logarithmic scale.

liposomal L-CPT I displayed similar characteristics to the native enzyme in rat liver mitochondria. Once again, the greatest disparity was observed with the enzyme in *P. pastoris* mitochondria, where the curve appeared to saturate much more slowly than was the case for the other preparations. Under the assay conditions used (in the presence of 1% BSA) CPT displays non-ideal kinetics with regard to palmitoyl-CoA, making it impossible to calculate a meaningful K_m for that substrate. However, for comparative purposes, the palmitoyl-CoA responses are summarized in Table 2 as the concentration of substrate giving half the maximal activity observed at 300 μ M ($S_{300/2}$).

Figure 3 shows activity for each of the L-CPT I preparations over the pH range 5.6–8.8 (Figure 3A, solid lines). Similar profiles were obtained with fed and fasted liver mitochondria and with the reconstituted protein. In contrast, the enzyme in *P. pastoris* mitochondria showed markedly altered behaviour, appearing to have a lower pH optimum and to be more sensitive to

higher pH than the others. The dashed lines in Figure 3(A) represent similar experiments performed in the presence of 5 μ M malonyl-CoA. At our standard assay pH of 7.2, consistent with the data in Figure 2, the different preparations of L-CPT I showed a range of inhibition levels, the enzyme in fed liver mitochondria being the most sensitive while L-CPT I reconstituted at 4 °C was the least inhibited. In Figure 3(B), activity in the presence of 5 μ M malonyl-CoA is expressed as a percentage of that in the absence of the inhibitor. In all cases, malonyl-CoA became increasingly effective as the pH was lowered, consistent with earlier findings [31,32]. However, the slope of the curve in the pH range 6.5–7.5 varied markedly, fed liver showing the sharpest dependence of malonyl-CoA sensitivity on pH and liposomes generated at 22 °C being the least affected. At pH 8.0–8.8 malonyl-CoA was largely ineffective.

To assess the direct influence of membrane fluidity on the sensitivity of L-CPT I to malonyl-CoA, inhibition curves were obtained for the proteoliposomes after pretreatment with benzyl alcohol. The concentration of this agent (40 mM) and the time of exposure (8 min) have been shown to elicit a maximal effect on L-CPT I *in situ* in rat liver outer mitochondrial membranes [33]. As shown in Figure 4, fluidization of the artificial membrane environment caused a right-shift in the inhibition curve. This change (presented for clarity on a logarithmic scale) represents more than a doubling of the IC_{50} for proteoliposomes made at 22 °C (Figure 4A), the value increasing from 11.4 ± 2.0 to 26.7 ± 7.6 μ M. Proteoliposomes generated with the 4 °C protocol, already less sensitive compared with those manufactured at the higher temperature, were also capable of further desensitization with benzyl alcohol (Figure 4B). In this case, the IC_{50} rose from 34.3 ± 6.1 to 67.7 ± 7.5 μ M. Total activities in the absence of malonyl-CoA were also increased slightly by the fluidization protocol [values after treatment of 121 ± 6 and 113 ± 9 % relative to untreated controls, for liposomes produced at 4 and 22 °C, respectively ($n = 3$ in each case)].

DISCUSSION

Our primary goal here was to establish a protocol by means of which a mammalian mitochondrial CPT I isoenzyme could be obtained in pure and active form and with characteristics as close as possible to those of the enzyme in its natural environment. We chose to work with rat L-CPT I for three reasons. First, to permit direct comparison with the native protein, we preferred to work with a rat enzyme, since mitochondria from this species are readily available. Second, the enzyme as it exists in rat liver mitochondria has been studied extensively [1]. Third, we were particularly interested in a unique property of the L-isoform of CPT I, namely its changing sensitivity to malonyl-CoA under different metabolic circumstances.

In previous structure–function studies of the CPT enzymes we have used *Escherichia coli*, *S. cerevisiae* and mammalian COS cells as expression vehicles [11,12,23,24,29,34,35]. Each has its advantages. Whereas COS cells provide a mammalian mitochondrial environment for the expressed protein, they also contain an endogenous CPT activity that can complicate interpretation of the data. In contrast, the prokaryotic and yeast systems have no baseline CPT activity, and both have proved useful for expression of CPT II [29,35]. However, recombinant L-CPT I was produced only at low levels by *S. cerevisiae* [24]. By contrast, all three mitochondrial CPT isoforms, i.e. L-CPT I, M-CPT I and CPT II, were found to be expressed at high levels in *P. pastoris* [25,27,36–39]. In the present work, not only did this yeast strain synthesize the 6 $\times$ His-tagged rat L-CPT I efficiently, but purification of the recombinant product using Ni-NTA–

agarose was accomplished with relative ease and rapidity, and the yield of ≈ 2 mg of purified protein/l of *P. pastoris* culture (high for an integral membrane protein) was ample for our purposes.

As would be predicted from earlier studies with L-CPT I in rat liver mitochondria [28] or the protein expressed in *S. cerevisiae* [24], the detergent-solubilized enzyme produced by *P. pastoris* possessed very little catalytic activity. However, due to the highly purified and concentrated nature of our L-CPT I preparation, even with the very low specific activity of the solubilized enzyme, we could determine that the activity present was not sensitive to malonyl-CoA. Woldegiorgis and colleagues have previously shown that when L-CPT I is expressed in *P. pastoris*, a Triton X-100 extract of total membrane protein/lipid can be combined with additional phospholipid *in vitro* with partial restoration of catalytic activity [36]. Using a similar approach, but this time using a highly purified and lipid-free preparation of L-CPT I, when our enzyme was combined with phospholipids and the detergent removed with an adsorbent, its specific activity increased some 200–400-fold and, importantly, it proved to be malonyl-CoA-sensitive. In addition, the reconstituted material displayed properties characteristic of proteoliposomes. This is the first time that a catalytically active and malonyl-CoA-sensitive CPT I enzyme has been obtained in pure form. Furthermore, as discussed in more detail below, the substrate kinetics and pH dependence of the reconstituted, 6 $\times$ His-tagged L-CPT I were similar to those of the enzyme in rat liver mitochondria.

This advance allowed us to assess the kinetic characteristics of L-CPT I in a variety of membrane environments. Specifically, we compared the properties of the purified enzyme reconstituted by two slightly different protocols with those of the protein as it exists when over-expressed in the *P. pastoris* mitochondrial membrane and, importantly, with those of the native protein in liver mitochondria taken from fed and fasted rats. The data for malonyl-CoA sensitivity of L-CPT I in intact liver mitochondria illustrate the well-established phenomenon of the enzyme's desensitization to the inhibitor that occurs with fasting. Although this observation has been made many times in different laboratories, understanding the mechanism of the change has been a major challenge and several theories have been proposed. Kolodziej and Zammit showed that when chemical reagents were used to artificially increase the fluidity of rat liver mitochondrial membranes L-CPT I became less sensitive to inhibition [33]. Also, use of fluorescent probes indicated that conditions under which desensitization of hepatic L-CPT I occurs *in vivo* are associated with decreased order of the hydrophobic core of the membrane [19]. It was suggested, therefore, that the state of the membrane might significantly influence the properties of the enzyme. However, in the same study, no consistent correlation could be made between changes in L-CPT I sensitivity and gross alterations in the lipid composition of the membrane. It was proposed that variations in lipid make-up could occur at the level of 'microdomains' within the outer membrane. In that context, it is interesting to note that CPT I may be concentrated at the mitochondrial membrane contact sites [40].

The idea has also been put forward that the prevailing level of malonyl-CoA itself might play a direct role, a concept suggested by the finding that pre-incubation of mitochondria with malonyl-CoA can enhance subsequent sensitivity of L-CPT I to the inhibitor [41,42]. Consistent with this notion is that physiological conditions associated with a decreased sensitivity are also those in which the malonyl-CoA levels are lowest. More recently, it has been proposed that the apparent change in sensitivity on fasting may not result from a conformational change in the existing L-CPT I protein, but from the induced expression of a less-sensitive

CPT I isoform [15]. Yet another possibility is that the change is the consequence of interaction of CPT I with some as-yet-unidentified regulatory protein. The protocol we have developed provided a previously unavailable purified enzyme model with which to examine the phenomenon. In particular, we have been able for the first time to study variation in the properties of the same enzyme in various environments and under conditions devoid of any putative specifically interacting regulatory proteins and in some cases entirely outside the mitochondrial milieu. This approach has permitted important new light to be shed on the likely mechanism of desensitization to malonyl-CoA.

Initial comparison of the recombinant L-CPT I over-expressed in *P. pastoris* with the native protein in liver mitochondria clearly demonstrates that the properties of both are broadly similar. In terms of malonyl-CoA sensitivity, L-CPT I present in *P. pastoris* mitochondria behaved most like the desensitized, 'fasted' version of the enzyme *in vivo*. Others have reported IC_{50} values for L-CPT I in *P. pastoris* as low as 1.9 μ M [36] and as high as 54 μ M [39]. The reasons for these discrepancies are unknown, but presumably reflect some differences in the details of the expression system, handling of the protein by the yeast cells under different circumstances, sample preparation or CPT assay. Hence, simple comparison of the sensitivity of the enzyme over-expressed in the yeast cell with those of L-CPT I *in situ* in the complex protein milieu of the rat liver mitochondrial outer membrane provides little clarification of the desensitization phenomenon. We were interested in determining if the membrane lipid environment could have a direct effect on the enzyme's response to malonyl-CoA. The critical observation made here is that the same recombinant L-CPT I protein that displays a certain sensitivity to malonyl-CoA when present in the *P. pastoris* mitochondrial membrane may display a similar, or markedly lower, sensitivity when reconstituted into the artificial liposomes, depending only on a slight variation in the reconstitution protocol. The physical explanation for the effect of temperature on the reconstitution is unclear, but presumably reflects differences in the structure of the liposomes formed. Although primarily phosphatidylcholine, soya bean asolectin contains a mixture of phospholipids, varying in both head group and lipid moieties, and therefore it is not surprising that the differential phase behaviours of these compounds should lead to temperature-dependent variation in the make up of the liposomes. It should be noted that desensitization of L-CPT I in liver mitochondria can occur with partial proteolysis [42,43], presumably through loss of the N-terminal domain. However, we do not believe that significant degradation of our protein occurred. Certainly, SDS/PAGE analysis of the reconstituted enzyme shows no evidence for proteolysis and, furthermore, the IC_{50} of 30 μ M for the least-sensitive liposomal enzyme is much lower than that observed (80 μ M) for an N-terminally truncated form of the protein [24]. Furthermore, it is the 4 °C reconstitution protocol, during which any proteolysis would be expected to be relatively slow, that yields the less sensitive product. We believe, therefore, that the identical polypeptide can assume active conformations with different levels of sensitivity. Additional evidence for this proposition comes from the experiments with benzyl alcohol. Pre-treatment of rat liver outer mitochondrial membranes with the membrane-fluidizing agent had been shown to result in a relative desensitization of L-CPT I *in vitro* [33]. However, in that study, the possibility could not be excluded that the effects of changing membrane fluidity were mediated by interaction with other proteins in the mitochondrial membrane. Since we were able to reproduce this effect in the purified system, it is evident that the physical properties of the membrane/liposome directly influence the conformation of the protein in such a way as to affect malonyl-CoA sensitivity.

This is consistent with the lipid 'microdomain' theory proposed by Zammit and co-workers ([19,33], discussed above). While not immediately pertinent to the current work, it will be interesting to determine in later studies if the muscle isoform of CPT I, which does not appear to alter its malonyl-CoA sensitivity *in vivo*, can be induced to do so in an *in vitro* system.

Current understanding of CPT I topology holds that the N-terminal domain (essential for the malonyl-CoA response) and the C-terminal 'catalytic' domain both lie on the outside of the mitochondrial outer membrane, connected by a short loop in the inter-membrane space [20–23]. This model provides for an attractive, though admittedly speculative, scenario in which it is alteration in the interaction between these two domains, rather than changes within either one, that results in modulation of malonyl-CoA sensitivity. Since it is the membrane that anchors these two domains in juxtaposition, it is not surprising that changes in the physical properties of the membrane could produce the observed effects. This could also explain why the detergent-solubilized enzyme has no apparent sensitivity to the inhibitor. Such a formulation would predict that anything that affects the malonyl-CoA response need not affect the properties of the active site, and vice versa. This is also supported by the kinetic data presented here. Thus we find that the reconstituted enzyme, which displays variation in sensitivity to malonyl-CoA, retains catalytic characteristics (in terms of its interaction with the substrates) that are most similar to the enzyme in rat liver mitochondria. In contrast, L-CPT I in *P. pastoris* mitochondria, while maintaining an inhibitor sensitivity similar to that of the native rat enzyme, appears to diverge from it in its responses to carnitine and palmitoyl-CoA. This separation of catalytic and inhibitory properties is most clearly illustrated by the finding that in the absence of malonyl-CoA the pH profile for the CPT I reaction is essentially the same for the enzyme in 'fed' mitochondria, 'fasted' mitochondria and liposomes. On the other hand, once a low level of malonyl-CoA is introduced, the profiles for each of the L-CPT I preparations differ radically. The early observation that the effectiveness of malonyl-CoA as an inhibitor decreases dramatically as the pH is raised from 6.4 to 7.6 suggested that a histidine residue might be involved in the regulatory interaction with malonyl-CoA, since it would be expected to titrate over this range [31,32]. Indeed certain candidate histidines have been identified [23]. However, a detailed kinetic analysis of the mitochondrial enzyme has indicated that the interaction with malonyl-CoA has two components [44]. These most probably represent inhibition via a specific, allosteric binding site, probably involving an N-terminal histidine residue and responsible for the primary inhibitory action, and a secondary, minor inhibition effected through direct competition with the acyl-CoA substrate at the active site. Because the latter would not be predicted to have such a sharp dependence on pH, it follows that as the relationship between the two domains becomes less strong, the competitive component would account for a progressively larger proportion of the inhibition observed. As a result, the effect of pH lowering (to enhance the inhibitory effect of malonyl-CoA) should be diminished. The fact that the pH dependence of the effectiveness of a fixed concentration of malonyl-CoA decreased in the order of fed liver mitochondria > fasted liver mitochondria ≈ liposomes made at 22 °C ≈ *P. pastoris* mitochondria > liposomes made at 4 °C, and that this sequence was reversed in terms of the IC₅₀ of L-CPT I for malonyl-CoA at pH 7.2, suggests that the strength of interaction between the N- and C-terminal domains of the protein also decreased in the order shown.

We conclude that the kinetic properties of L-CPT I can vary widely depending upon the membrane environment and in-

dependently of modification of the protein or its interaction with regulatory subunits. It is probable that this explains much of the variation that is observed *in vivo*. However, although alterations in the membrane may be sufficient to elicit changes in the enzyme seen *in vivo*, this does not exclude a role for other proteins in such regulation. Also, the possibility that alternative CPT I isoforms might be induced in the liver during a fast is not disproved by the present work [15]. However, that theory awaits direct demonstration of the existence of the splice variants at the protein level.

Finally, the model described provides the first opportunity to study a CPT I enzyme in a pure and active form. It has already yielded important new information about how the catalytic and allosteric properties of L-CPT I may be related and how they are influenced by the interaction of the protein with the membrane. To date, the lack of purified, active protein has severely hampered progress in CPT I structure/function research. In addition to the insights it has already provided, we believe that purified, liposomal CPT I will provide an invaluable tool to investigate the structure-function and regulatory properties of this complex enzyme that plays such a central role in the regulation of fuel metabolism.

This work was supported by a grant from the National Institutes of Health (DK18573).

REFERENCES

- McGarry, J. D. and Brown, N. F. (1997) The mitochondrial carnitine palmitoyltransferase system: from concept to molecular analysis. *Eur. J. Biochem.* **244**, 1–14
- McGarry, J. D., Mills, S. E., Long, C. S. and Foster, D. W. (1983) Observations on the affinity for carnitine, and malonyl-CoA sensitivity, of carnitine palmitoyltransferase I in animal and human tissues: demonstration of the presence of malonyl-CoA in non-hepatic tissues of the rat. *Biochem. J.* **214**, 21–28
- Duan, C. and Winder, W. W. (1992) Nerve stimulation decreases malonyl-CoA in skeletal muscle. *J. Appl. Physiol.* **72**, 901–904
- Odland, L. M., Heigenhauser, G. J. F., Lopaschuk, G. D. and Spriet, L. L. (1996) Human skeletal muscle malonyl-CoA at rest and during prolonged submaximal exercise. *Am. J. Physiol.* **270**, E541–E544
- Prentki, M. and Corkey, B. E. (1996) Are the β -cell signaling molecules malonyl-CoA and cytosolic long-chain acyl-CoA implicated in multiple tissue defects of obesity and NIDDM? *Diabetes* **45**, 273–283
- Foley, J. E. (1992) Rationale and application of fatty acid oxidation inhibitors in treatment of diabetes mellitus. *Diabetes Care* **15**, 773–784
- Deems, R. O., Anderson, R. C. and Foley, J. E. (1998) Hypoglycemic effects of a novel fatty acid oxidation inhibitor in rats and monkeys. *Am. J. Physiol.* **274**, R524–R528
- Lopaschuk, G. D., Belke, D. D., Gamble, J., Itoi, T. and Shonekess, B. O. (1994) Regulation of fatty acid oxidation in the mammalian heart in health and disease. *Biochim. Biophys. Acta* **1213**, 263–276
- Stanley, W. C., Lopaschuk, G. D., Hall, J. L. and McCormack, J. G. (1997) Regulation of myocardial carbohydrate metabolism under normal and ischemic conditions: potential for pharmacological interventions. *Cardiovasc. Res.* **33**, 243–257
- Kantor, P. F., Dyck, J. R. and Lopaschuk, G. D. (1999) Fatty acid oxidation in the reperfused ischemic heart. *Am. J. Med. Sci.* **318**, 3–14
- Esser, V., Britton, C. H., Weis, B. C., Foster, D. W. and McGarry, J. D. (1993) Cloning, sequencing, and expression of a cDNA encoding rat liver carnitine palmitoyltransferase I: direct evidence that a single polypeptide is involved in inhibitor interaction and catalytic function. *J. Biol. Chem.* **268**, 5817–5822
- Esser, V., Brown, N. F., Cowan, A. T., Foster, D. W. and McGarry, J. D. (1996) Expression of a cDNA isolated from rat brown adipose tissue and heart identifies the product as the muscle isoform of carnitine palmitoyltransferase I (M-CPT-I): M-CPT I is the predominant CPT I isoform expressed in both white (epididymal) and brown adipocytes. *J. Biol. Chem.* **271**, 6972–6977
- Yu, G.-S., Lu, Y.-C. and Gulick, T. (1998) Rat carnitine palmitoyltransferase I/ β mRNA splicing isoforms. *Biochim. Biophys. Acta* **1393**, 166–172
- Yu, G.-S., Lu, Y.-C. and Gulick, T. (1998) Expression of novel isoforms of carnitine palmitoyltransferase I (CPT I) generated by alternative splicing of the CPT I/ β gene. *Biochem. J.* **334**, 225–231
- Yu, G.-S., Lu, Y.-C. and Gulick, T. (1998) Co-regulation of tissue specific alternative human carnitine palmitoyltransferase I/ β gene promoters by fatty acid enzyme substrate. *J. Biol. Chem.* **273**, 32901–32909

- 16 Yamazaki, N., Shinohara, Y., Shima, A. and Terada, H. (1995) High expression of a novel carnitine palmitoyltransferase I like protein in rat brown adipose tissue and heart: isolation and characterization of its cDNA clone. *FEBS Lett.* **363**, 41–45
- 17 Ghadiminejad, I. and Saggerson, E. D. (1991) A study of properties and abundance of the components of liver carnitine palmitoyltransferases in mitochondrial inner and outer membranes. Effects of hypothyroidism, fasting and a ketotic diabetic state. *Biochem. J.* **277**, 611–617
- 18 Zammit, V. A. (1996) Role of insulin in hepatic fatty acid partitioning: emerging concepts. *Biochem. J.* **314**, 1–14
- 19 Zammit, V. A., Corstorphine, C. G., Kolodziej, M. P. and Fraser, F. (1998) Lipid molecular order in liver mitochondrial outer membranes, and sensitivity of carnitine palmitoyltransferase I to malonyl-CoA. *Lipids* **33**, 371–376
- 20 Kolodziej, M. P. and Zammit, V. A. (1993) Mature carnitine palmitoyltransferase I retains the N-terminus of the nascent protein in rat liver. *FEBS Lett.* **327**, 294–296
- 21 Fraser, F., Corstorphine, C. G. and Zammit, V. A. (1996) Evidence that both the acyl-CoA- and malonyl-CoA binding sites of mitochondrial overt carnitine palmitoyltransferase (CPT I) are exposed on the cytosolic face of the outer membrane. *Biochem. Soc. Trans.* **24**, 184S
- 22 Fraser, F., Corstorphine, C. G. and Zammit, V. A. (1997) Topology of carnitine palmitoyltransferase I in the mitochondrial outer membrane. *Biochem. J.* **323**, 711–718
- 23 Swanson, S. T., Foster, D. W., McGarry, J. D. and Brown, N. F. (1998) Roles of the N- and C-terminal domains of carnitine palmitoyltransferase I isoforms in malonyl-CoA sensitivity of the enzymes: insights from expression of chimeric proteins and mutation of conserved histidine residues. *Biochem. J.* **335**, 513–519
- 24 Brown, N. F., Esser, V., Foster, D. W. and McGarry, J. D. (1994) Expression of a cDNA for rat liver carnitine palmitoyltransferase I in yeast establishes that catalytic activity and malonyl-CoA sensitivity reside in a single polypeptide. *J. Biol. Chem.* **269**, 26438–26442
- 25 Shi, J., Zhu, H., Arvidson, D. N., Cregg, J. M. and Woldegiorgis, G. (1998) Deletion of the first 18 N-terminal amino acid residues in rat liver carnitine palmitoyltransferase I abolishes malonyl-CoA sensitivity and binding. *Biochemistry* **37**, 11033–11038
- 26 Cohen, I., Kohl, C., McGarry, J. D., Girard, J. and Prip-Buus, C. (1998) The N-terminal domain of rat liver carnitine palmitoyltransferase I mediates import into the outer mitochondrial membrane and is essential for activity and malonyl-CoA sensitivity. *J. Biol. Chem.* **273**, 29896–29904
- 27 Shi, J., Zhu, H., Arvidson, D. N. and Woldegiorgis, G. (1999) A single amino acid change (substitution of glutamate 3 with alanine) in the N-terminal region of rat liver carnitine palmitoyltransferase I abolishes malonyl-CoA inhibition and high affinity binding. *J. Biol. Chem.* **274**, 9421–9426
- 28 Woeltje, K. F., Kuwajima, M., Foster, D. W. and McGarry, J. D. (1987) Characterization of the mitochondrial carnitine palmitoyltransferase enzyme system. II. Use of detergents and antibodies. *J. Biol. Chem.* **262**, 9822–9827
- 29 Brown, N. F., Anderson, R. C., Caplan, S. L., Foster, D. W. and McGarry, J. D. (1994) Catalytically important domains of rat carnitine palmitoyltransferase II as determined by site-directed mutagenesis and chemical modification: evidence for a critical histidine residue. *J. Biol. Chem.* **269**, 19157–19162
- 30 Garlid, K. D., Sun, X., Paucek, P. and Woldegiorgis, G. (1995) Mitochondrial cation transport systems. *Methods Enzymol.* **260**, 331–348
- 31 Stephens, T. W., Cook, G. A. and Harris, R. A. (1983) Effect of pH on malonyl-CoA inhibition of carnitine palmitoyltransferase I. *Biochem. J.* **212**, 521–524
- 32 Mills, S. E., Foster, D. W. and McGarry, J. D. (1984) Effect of pH on the interaction of substrates and malonyl-CoA with mitochondrial carnitine palmitoyltransferase I. *Biochem. J.* **219**, 601–608
- 33 Kolodziej, M. P. and Zammit, V. A. (1990) Sensitivity of inhibition of rat liver mitochondrial outer-membrane carnitine palmitoyltransferase by malonyl-CoA to chemical- and temperature-induced changes in membrane fluidity. *Biochem. J.* **272**, 421–425
- 34 Woeltje, K. F., Esser, V., Weis, B. C., Sen, A., Cox, W. F., McPhaul, M. J., Slaughter, C. A., Foster, D. W. and McGarry, J. D. (1990) Cloning, sequencing, and expression of a cDNA encoding rat liver mitochondrial carnitine palmitoyltransferase II. *J. Biol. Chem.* **265**, 10720–10725
- 35 Brown, N. F., Sen, A., Soltis, D. A., Jones, B., Foster, D. W. and McGarry, J. D. (1993) Expression of precursor and mature carnitine palmitoyltransferase II in *Escherichia coli* and *in vitro*: differential behaviour of rat and human isoforms. *Biochem. J.* **294**, 79–86
- 36 de Vries, Y., Arvidson, D. N., Waterham, H. R., Cregg, J. M. and Woldegiorgis, G. (1997) Functional characterization of mitochondrial carnitine palmitoyltransferases I and II expressed in the yeast *Pichia pastoris*. *Biochemistry* **36**, 5285–5292
- 37 Zhu, H., Shi, J., Cregg, J. M. and Woldegiorgis, G. (1997) Reconstitution of highly expressed human heart muscle carnitine palmitoyltransferase I. *Biochem. Biophys. Res. Commun.* **239**, 498–502
- 38 Zhu, H., Shi, J., de Vries, Y., Arvidson, D. N., Cregg, J. M. and Woldegiorgis, G. (1997) Functional studies of yeast-expressed human heart muscle carnitine palmitoyltransferase I. *Arch. Biochem. Biophys.* **347**, 53–61
- 39 Jackson, V. N., Cameron, J. M., Zammit, V. A. and Price, N. T. (1999) Sequencing and functional expression of the malonyl-CoA-sensitive carnitine palmitoyltransferase from *Drosophila melanogaster*. *Biochem. J.* **341**, 483–489
- 40 Fraser, F. and Zammit, V. A. (1998) Enrichment of carnitine palmitoyltransferases I and II in the contact sites of rat liver mitochondria. *Biochem. J.* **329**, 225–229
- 41 Zammit, V. A. and Corstorphine, C. G. (1985) Effects of incubation at physiological temperatures on the concentration-dependence of [$^2\text{-}^{14}\text{C}$]malonyl-CoA binding to rat liver mitochondria. *Biochem. J.* **231**, 343–347
- 42 McGarry, J. D., Brown, N. F., Inthanousay, P. P., Park, D. I., Cook, B. A. and Foster, D. W. (1992) Insights into the topography of mitochondrial carnitine palmitoyltransferase gained from the use of proteases, in *New Developments in Fatty Acid Oxidation* (Coates, P. M. and Tanaka, K., eds.), pp. 47–61, Wiley-Liss, New York
- 43 Kashfi, K. and Cook, G. A. (1995) Temperature effects on malonyl-CoA inhibition of carnitine palmitoyltransferase I. *Biochim. Biophys. Acta* **1257**, 133–139
- 44 Cook, G. A., Mynatt, R. L. and Kashfi, K. (1994) Yonetani-Theorell analysis of hepatic carnitine palmitoyltransferase-I inhibition indicates two distinct inhibitory binding sites. *J. Biol. Chem.* **269**, 8803–8807