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# Distinct Kinetics of Carnitine Palmitoyltransferase I in Contact Sites and Outer Membranes of Rat Liver Mitochondria*

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**Carnitine palmitoyltransferase I (CPT I) of rat liver mitochondria is an integral, polytopic protein of the outer membrane that is enriched at contact sites. As CPT I kinetics are highly dependent on its membrane environment, we have measured the kinetic parameters of CPT I present in rat liver submitochondrial membrane fractions enriched in either outer membrane or contact sites. The $K_m$ for palmitoyl-CoA was 2.4-fold higher for CPT I in outer membranes than that for the enzyme in contact sites. In addition, whereas in contact sites malonyl-CoA behaved as a competitive inhibitor of CPT I with respect to palmitoyl-CoA, in outer membranes malonyl-CoA inhibition was non-competitive. As a result of the combination of these changes, the $IC_{50}$ for malonyl-CoA was severalfold higher for CPT I in contact sites than for the enzyme in bulk outer membrane. The $K_i$ for malonyl-CoA, the $K_m$ for carnitine, and the catalytic constant of the enzyme were all unaffected. It is concluded that the different membrane environments in outer membranes and contact sites result in an altered conformation of L-CPT I that specifically affects the long-chain acyl-CoA binding site. The accompanying changes in the kinetics of the enzyme provide an additional potent mechanism for the regulation of L-CPT I activity.**

The overt carnitine palmitoyltransferase of mitochondria (CPT I)[1] catalyzes the rate-limiting step in the transfer of acyl moieties from the cytosolic compartment into the mitochondrial matrix, where they undergo β-oxidation. The enzyme is an integral membrane protein with two transmembrane segments that are thought to confer on its kinetics a marked dependence on the physical state of the membrane (1–4). CPT I is present within the general outer membrane of mitochondria (5) but is especially concentrated within the contact sites that occur between the outer membrane and the peripheral inner membrane (6). The latent form of carnitine palmitoyltransferase (CPT II) is also concentrated at the contact sites but on the inner aspect of the inner membrane, suggesting that long-chain acylcarnitine formation, and utilization may be facilitated by this submitochondrial localization of the two proteins (6). Contact sites are loci for the extensive trafficking of proteins and phospholipids between the extra- and intramitochondrial compartments. They are the sites of the mitochondrial protein import machinery (7) of the binding of hexokinase and creatine kinase on the cytosolic and intermembrane space aspects of the outer membrane, respectively (8, 9) and of the attachment of a specialized population of endoplasmic reticular membranes through which phospholipid trafficking occurs (10, 11). They are also sites of interaction of mitochondria with the cytoskeleton (12).

The property that makes CPT I rate-limiting for long-chain acyl-CoA utilization by mitochondria is its inhibition by malonyl-CoA (13). The mechanism of this inhibition, as studied in intact isolated mitochondria, appears to be competitive with respect to the acyl-CoA substrate. However, it has long been appreciated that its mode of action is unorthodox as malonyl-CoA appears to induce sigmoidicity in the velocity-substrate concentration curves (14). CPT I kinetics are very sensitive to changes in the physical properties of the membrane in which it resides (3). Fluidization of membrane lipids *in vivo* or *in vitro* results in the desensitization of CPT I to malonyl-CoA and vice versa (3, 15, 16). We have reasoned, therefore, that as contact sites have a distinctive lipid (17) and protein (18) composition, it is possible that CPT I molecules in the two membrane microenvironments may have different kinetic properties (6) as is already well established for other proteins (*e.g.* porin; see Refs. 19 and 20) that exist in both outer membrane and contact site environments.

We have tested this hypothesis by studying the kinetics of liver CPT I in submitochondrial fractions enriched in the two membrane populations. We find that the kinetic parameters with respect to one of its substrates, palmitoyl-CoA, are markedly different for the enzyme in the two microenvironments. Moreover, the kinetics of malonyl-CoA inhibition of CPT I activity is different for the enzyme resident within the two membrane populations.

## MATERIALS AND METHODS

*Preparation and Subfractionation of Mitochondria*—Liver mitochondria were prepared from male Wistar rats (350–400-g body weight; maintained on a laboratory chow diet) by differential centrifugation followed by Percoll gradient purification. Mitochondrial subfractionation was performed as described previously (6). However, the sonicated mitochondria were fractionated using step, rather than continuous, sucrose gradients. The sucrose concentrations for the step gradient were 1.13 g, 1.15 g, and 1.19 g/ml in 10 mM potassium phosphate buffer, pH 7.0, and chosen to yield fractions enriched in outer membrane, contact sites, and inner membrane, respectively (6). The volume of each sucrose density step was 3 ml. A step (1.5-ml) of intermediate density was present between the 1.13-g and 1.15 g/ml steps to ensure clean separation between the outer membrane and contact site fractions. The gradients were centrifuged at 100,000 × *g* for 210 min at 4 °C in a 65V13 vertical rotor (Sorvall Instruments). The fractions were recovered by puncturing the polycarbonate tubes with a syringe needle followed by aspiration of each membrane band. They were divided into

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[1] The abbreviation used is: CPT overt, carnitine palmitoyltransferase of mitochondria.


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TABLE I

*Characterization of representative outer membrane and contact site fractions*

Mitochondria were prepared from livers of fed rats, and submitochondrial fractions were isolated on discontinuous sucrose gradients as described in the "Materials and Methods." The activities of marker enzymes for outer membrane (rotenone-insensitive NADPH cytochrome *c* reductase; rNCR) and inner membrane (cytochrome *c* oxidase) were measured and are given as μmol/min per total fraction. The abundance of porin (marker for outer membrane and contact sites) and of CPT I in each fraction was measured by phosphorimage analysis of Western blots obtained using appropriate primary and secondary antibodies (see "Materials and Methods"). In both instances the intensity of the immunoreactive bands was normalized by setting that obtained for the outer membrane fraction at unity, for any one preparation, and expressing the other fractions as a proportion. Values are means of five different preparations.

| Protein | Outer membrane | Contact sites | Inner membrane |
|---|---|---|---|
| rNCR | 20.31 ± 3.17 | 5.00 ± 0.76 | 2.44 ± 0.52 |
| Cytochrome *c* oxidase | 4.30 ± 1.10 | 49.25 ± 21.61 | 72.97 ± 20.70 |
| Porin | 1 ± 0.12 | 0.42 ± 0.09 | 0.11 ± 0.05 |
| CPT I | 1 ± 0.33 | 0.49 ± 0.19 | 0.02 ± 0.01 |

aliquots and frozen at −20 °C until used for enzyme activity measurements, protein determination, and SDS polyacrylamide gel electrophoresis.

*Marker Protein Determination*—The activities of marker enzymes were measured to characterize the purity of the fractions. Rotenone-insensitive NADPH-cytochrome *c* reductase and cytochrome *c* oxidase (assayed as in Ref. 6) were used as markers for outer and inner membrane fractions, respectively. Porin was used as a marker for both outer membrane and contact sites (6). The component proteins of the submitochondrial fractions were separated using SDS polyacrylamide gel electrophoresis (15% polyacrylamide) followed by transfer onto nitrocellulose and immunodetection of porin with mouse anti-human porin conjugated to alkaline phosphatase (see Ref. 6). The band intensity of porin on the Western blots was quantified densitometrically (Molecular Dynamics).

*Assay of Activity and Immunodetection of CPT I*—In Ref. 6 we showed that CPT II is also enriched within contact sites and that some contamination of the outer membranes with CPT II is always present. Therefore, samples of the sucrose gradient-purified submitochondrial fractions enriched in outer membranes or contact sites were preincubated for 10 min on ice in the absence and presence of tetradecylglycidyl-CoA at a concentration sufficient to inhibit all CPT I activity. This enabled us to quantify the proportion of total CPT activity in these fractions that was due to CPT II in these fractions. This was then subtracted from the total CPT activity (obtained in fractions not incubated with the inhibitor) so as to obtain that of CPT I.

The basic assay medium for CPT I contained 80 mM sucrose, 70 mM KCl, 50 mM imadazole, 1 mM EGTA, 5 mM MgATP, 1% defatted bovine serum albumin, 2 mM glutathione, 4 μg of rotenone/ml, 2 μg of antimycin A/ml, and the indicated concentrations of carnitine (specific radioactivity 770 dpm/nmol) and palmitoyl-CoA. The final pH value was 7.1. For determination of the $IC_{50}$ for malonyl-CoA (concentration that gives 50% inhibition of CPT I activity) the concentrations of carnitine and palmitoyl-CoA were 520 and 35 μM, respectively. When the concentrations of carnitine or palmitoyl-CoA were varied, the fixed concentrations of the second substrate were 135 and 520 μM for palmitoyl-CoA and carnitine, respectively. The specific activity of $^3$H-carnitine was kept constant at 1077 dpm/nmol. Malonyl-CoA concentrations were either varied up to 200 μM or maintained constant at 20 μM when investigating the dependence of CPT I activity on increasing palmitoyl-CoA concentrations in the absence or presence of malonyl-CoA.

Immunodetection of CPT I was performed by Western blotting as described previously, using an anti-peptide antibody (anti-C) raised against a linear epitope from the catalytic domain of rat liver CPT I (1, 6, 21).

Palmitoyl-CoA, malonyl-CoA, and fatty acid-free albumin, co-factors, and substrates for enzyme assays were from Sigma; mouse anti-human porin antibody was from Calbiochem-Novabiochem. Tetradecylglycidyl-CoA was synthesized starting with the sodium salt of tetradecylglycidic acid (McNeil Pharmaceutical, Spring House, PA) by the method described in Ref. 22. Radiolabeled carnitine was from Amersham Pharmacia Biotech and was washed with water-saturated *n*-butanol before being used, to reduce radioactive contaminants. Sheep anti-rat L-CPT I anti-peptide L antibody was obtained as described previously (see Ref. 1). All other chemicals were from Merck.

## RESULTS

The outer membrane- and contact site-enriched fractions were characterized with respect to their content of marker proteins and immunodetectable CPT I (Table I). The sucrose step gradients used for their preparation in the present study gave fractions that had the characteristics of the respective peaks obtained from continuous gradients in our previous studies (6). There was minimal cross-contamination between the two fractions, although as expected this was greater for the contamination of contact sites with outer membrane (see Table I).

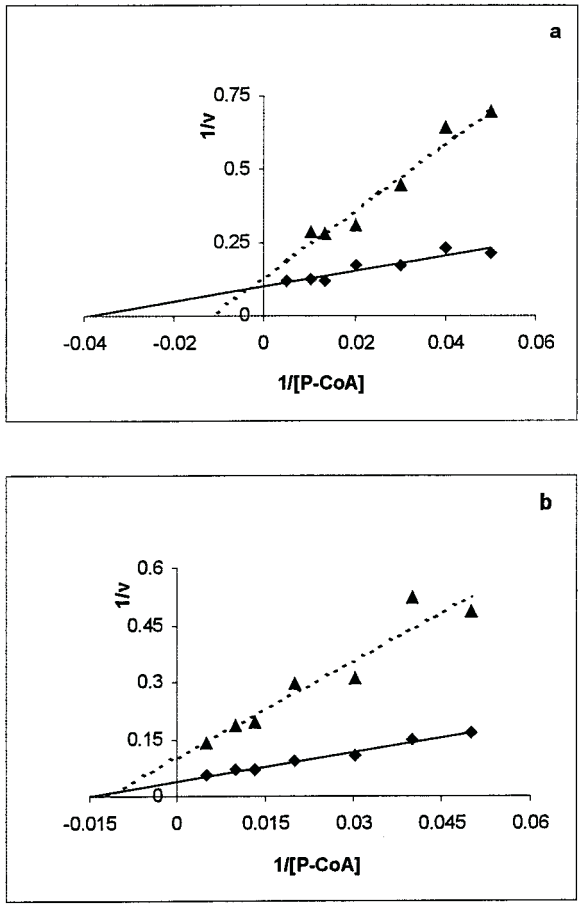


FIG. 1. **Lineweaver-Burke plots for the inhibition of CPT I by malonyl-CoA in (*a*) contact sites and (*b*) outer membranes.** Mitochondria were isolated, submitochondrial fractions were prepared as described in the "Materials and Methods," and CPT I activity was measured in the absence (♦) or presence of 20 μM malonyl-CoA (▲). Each pair of curves is representative of data obtained from four different preparations that gave similar results.

*$K_m$ for Palmitoyl-CoA and Carnitine*—Lineweaver-Burke plots for CPT I activity at a different palmitoyl-CoA concentration for the enzyme in contact site and outer membrane fractions were linear (see Fig. 1, *a* and *b*), as were Eadie-Hofstee plots (not shown), which allowed us to use graphical methods to determine the kinetic parameters. As shown in Table II, there was a 2.4-fold difference ($p = 0.0002$) in the $K_m$ for palmitoyl-CoA for the enzyme in the two different membrane fractions (60.6 ± 7.5 and 25.6 ± 0.9 μM for outer membrane and contact

TABLE II

*Kinetic parameters of CPT I in contact site and outer membrane fractions prepared from rat liver mitochondria*

Submitochondrial fractions were prepared from liver mitochondria of fed male rats as described in the "Materials and Methods," and kinetic parameters were determined for five different preparations. The $V_{max}$ and $K_m$ for palmitoyl-CoA were measured in the presence or absence of 20 $\mu$M malonyl-CoA as indicated. In the $V_{max}$ column the values are expressed per mg of protein of either contact sites or outer membranes. $IC_{50}$ values were obtained in the presence of 35 $\mu$M palmitoyl-CoA (plus 1% defatted albumin; see text). Values are means (± S.E.). Those that are statistically significantly different ($P < 0.001$) for the enzyme in the two membrane fractions are indicated by an asterisk.

| Fraction | Malonyl-CoA | $V_{max}$ | $K_{0.5}$ for palmitoyl-CoA | $K_m$ for carnitine | $K_i$ for malonyl-CoA | $IC_{50}$ for malonyl-CoA |
|---|---|---|---|---|---|---|
| | *20 $\mu$M* | *$\mu$mol/min/mg of membrane protein* | *$\mu$M* | *$\mu$M* | *$\mu$M* | *$\mu$M* |
| Outer membranes | − | 24.09 ± 1.10 | 58.3 ± 5.2 | 112.1 ± 8.6 | 9.2 ± 0.9 | 9.9 ± 2.5 |
| | + | 8.13 ± 1.55* | 62.3 ± 10.7 | − | − | − |
| Contact sites | − | 11.01 ± 1.76 | 26.1 ± 0.84* | 159.0 ± 20.2 | 9.1 ± 1.1 | 45.7 ± 8.7* |
| | + | 9.04 ± 0.98 | 99.9 ± 11.0* | − | − | − |

site fractions, respectively). The maximum palmitoyl-CoA concentration used was 200 $\mu$M (in the presence of 1% bovine serum albumin) to avoid the detergent properties of this molecule. There was no significant difference between the $K_m$ for carnitine values for the enzyme in the two membrane fractions (Table II).

*Malonyl-CoA Inhibition of CPT I: Effects of Membrane Location*—The double-reciprocal plots resulting from the measurement of CPT I activity at different palmitoyl-CoA concentrations in outer membranes and contact sites (Fig. 1, *a* and *b*) show that the kinetics of malonyl-CoA inhibition of CPT I with respect to palmitoyl-CoA were different for the enzyme in the two membrane fractions. In the outer membranes, malonyl-CoA acted exclusively by lowering the $V_{max}$ of the enzyme, without affecting the $K_m$ for palmitoyl-CoA. By contrast, in contact sites, malonyl-CoA inhibited CPT I activity almost entirely by raising the $K_m$ for palmitoyl-CoA without affecting the $V_{max}$ of the enzyme (there was a very minor effect on $V_{max}$, but this can be accounted for by unavoidable minor contamination of contact sites with outer membranes flanking the points of physical contact between the outer and inner membranes). Malonyl-CoA (20 $\mu$M) increased the $K_m$ for palmitoyl-CoA of CPT I in contact sites by 4-fold ($p > 0.0001$), whereas it had no effect on the $K_m$ of the enzyme in outer membranes. By contrast, 20 $\mu$M malonyl-CoA reduced the $V_{max}$ of the enzyme resident in outer membranes by 70% ($p = 0.0002$) without affecting that for the enzyme in contact sites. In neither fraction did malonyl-CoA induce any non-linearity in double-reciprocal plots. Note that the $V_{max}$ values refer to activity per mg of membrane protein of each individual membrane fraction and not of whole mitochondria.

*$IC_{50}$ and $K_i$ for Malonyl-CoA*—The $IC_{50}$ value for malonyl-CoA (the concentration of malonyl-CoA required to inhibit CPT I activity by 50% at a suboptimal concentration of palmitoyl-CoA) is the parameter that is most commonly measured to assess malonyl-CoA sensitivity of CPT I in intact mitochondria (23–25). When this type of experiment was performed, the inhibition curves obtained in the presence of increasing concentrations of malonyl-CoA were different for the enzyme in outer membranes and contact sites, respectively. The enzyme in contact sites was less sensitive to malonyl-CoA inhibition (Fig. 2) *i.e* it had the higher $IC_{50}$ value (see Table II).

In view of the fact that the $K_m$ for palmitoyl-CoA and the mode of inhibition by malonyl-CoA were found to be different between the two fractions, values for the $K_i$ for malonyl-CoA were also obtained from Dixon plots (1/v *versus* [malonyl-CoA]). As can be seen from Table II, the $K_i$ value for malonyl-CoA was the same for CPT I in outer membranes and contact sites suggesting that the difference in $IC_{50}$ was due solely to the difference in $K_m$ for palmitoyl-CoA between the enzyme in outer membranes and contact sites.

*The Catalytic Constant for CPT I in Outer Membranes and Contact Sites*—The catalytic constant of an enzyme is defined as the ratio of $V_{max}$ divided by the molar amount of enzyme protein, and is a measure of the intrinsic catalytic activity of the protein molecules. A quantitative estimate of the number of CPT I molecules in outer membrane and contact site fractions was obtained by immunodetection of CPT I on Western blots for paired fractions obtained from individual rat livers. The value of the $V_{max}$ for CPT I activity (Table II) divided by immunoreactive CPT I band intensity (Table I) was obtained for the two membrane fractions. For five different preparations, the ratio between the parameters for the two membrane fractions was not significantly different from unity (0.92 ± 0.18) indicating that the intrinsic catalytic activity of CPT I is not different in the two membrane microenvironments. In this respect, it is noteworthy that the effect of 20 $\mu$M malonyl-CoA on the catalytic efficiency ($V_{max}/K_m$) of CPT I in either of the two membrane fractions was identical (a 75% decrease) as can be calculated from the data in Table II.

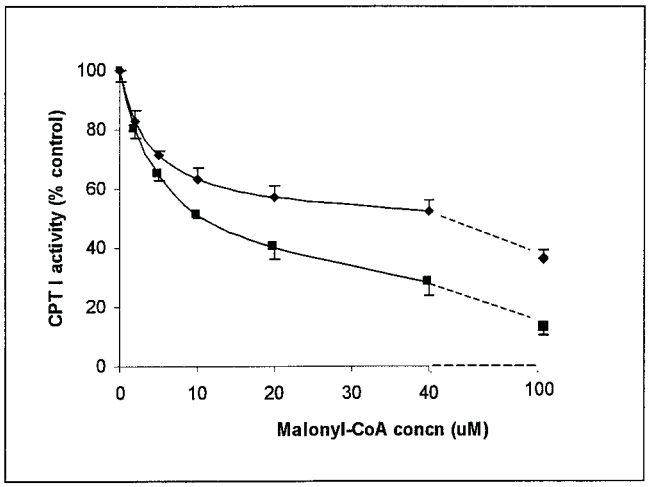


FIG. 2. **Inhibition of CPT I activity by malonyl-CoA in contact sites and outer membranes of rat liver mitochondria.** Contact site (◆) and outer membrane (■) fractions were prepared as described under "Materials and Methods." CPT I activity was assayed in the presence of fixed concentrations of palmitoyl-CoA (135 $\mu$M) and carnitine (520 $\mu$M) and varying concentrations of malonyl-CoA. Values are means (± S.E.).

## DISCUSSION

CPT I is an integral membrane protein that adopts a polytopic conformation (1). Its two hydrophobic transmembrane segments are flanked by the N- and C-terminal domains that both protrude into the cytosol. We have suggested (1, 2) that this topology endows the protein with the potential for altering the conformation of its catalytic C-domain in response to changes in the membrane environment. It was previously shown for mitochondria *in vitro* (15, 16) and for outer mem-

brane preparations isolated from rats in different physiological conditions (3) that CPT I kinetics are extremely sensitive to the molecular order of the constituent lipids of the membrane environment in which it resides or into which it is incorporated experimentally (4, 26). The observation (6) that CPT I is distributed both within the bulk outer membrane and contact sites (but enriched in the latter; see Ref. 6) raised the prospect that the kinetic properties of the enzyme are different in the two microenvironments. The present data provide evidence that this is indeed the case and that the properties of L-CPT I with respect to palmitoyl-CoA are markedly different in the two membrane environments. A possible mechanism for the changes observed could be the altered interaction of CPT I with other (lipid and/or protein) membrane components within outer membranes or contact sites resulting in a conformational change in the protein that affects specifically the acyl-CoA binding site. Previous observations (4, 27, 28) have shown that the kinetics of CPT I are highly dependent on the interaction between its cytosolic N-terminal domain and the rest of the molecule, as shown originally by cell biological studies (1, 2) and more recently by domain swapping (28) and functional mutagenesis experiments (27, 30). Therefore, it is plausible that short or long term changes in the membrane environment, because of either the formation of localized membrane microdomains of distinctive lipid and/or protein composition (*i.e.* in contact sites) or by changes in overall phospholipid composition (*e.g.* induced by diet, fasting, and/or insulin-deficiency; see Ref. 3) would induce altered kinetic characteristics of CPT I.

There are precedents of other proteins that show differences in molecular properties of populations that are partitioned between mitochondrial contact sites and outer membranes. Thus, voltage-dependent anion channel (porin), an integral mitochondrial outer membrane protein that is also enriched in contact sites (19, 20), displays different transport properties depending on its submitochondrial membrane location. For example, porin-rich domains present at contact sites of brain mitochondria bind hexokinase, whereas porin within the bulk outer membrane does not (12). Moreover, contact site porin is more difficult to extract with detergents, suggesting altered protein-lipid and/or protein-protein interactions (29).

The most striking difference between CPT I in contact sites and outer membrane was observed for the kinetics of malonyl-CoA inhibition with respect to palmitoyl-CoA. They were competitive for the enzyme resident within contact sites but non-competitive within the outer membrane fraction. It is significant that this change was accompanied by a 2.4-fold increase in the $K_m$ for palmitoyl-CoA but no change in the intrinsic catalytic activity of the enzyme, its $K_i$ for malonyl-CoA, or its $K_m$ for carnitine. Therefore, the change in CPT I appears to be specific to the acyl-CoA binding site. This difference in kinetics is potentially very important for the function of CPT I in the two membrane environments *in vivo*. Thus, not only would CPT I in the outer membranes be more susceptible to inhibition by malonyl-CoA at any given palmitoyl-CoA concentration (lower $IC_{50}$), but the inhibitor would be able to affect the outer membrane enzyme activity even at high palmitoyl-CoA concentrations because of the non-competitive nature of the inhibition for the enzyme in outer membranes. Consequently, the effect of malonyl-Co would not be able to be overridden by high palmitoyl-CoA concentrations unless the CPT I molecules reside within the contact sites.

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