Membrane microenvironment regulation of carnitine palmitoyltranferases I and II

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Abstract

CPT (carnitine palmitoyltransferase) 1 and CPT2 regulate fatty acid oxidation. Recombinant rat CPT2 was isolated from the soluble fractions of bacterial extracts and expressed in *Escherichia coli*. The acyl-CoA chain-length-specificity of the recombinant CPT2 was identical with that of the purified enzyme from rat liver mitochondrial inner membranes. The K_m for carnitine for both the mitochondrial preparation and the recombinant enzyme was identical. In isolated mitochondrial outer membranes, cardiolipin (diphosphatidylglycerol) increased CPT1 activity 4-fold and the K_m for carnitine 6-fold. It decreased the K_i for malonyl-CoA inhibition 60-fold, but had no effect on the apparent K_m for myristoyl-CoA. Cardiolipin also activated recombinant CPT2 almost 4-fold, whereas phosphatidylglycerol, phosphatidylserine and phosphatidylcholine activated the enzyme 3-, 2- and 2-fold respectively. Most of the recombinant CPT2 was found to have substantial interaction with cardiolipin. A model is proposed whereby cardiolipin may hold the fatty-acid-oxidizing enzymes in the active functional conformation between the mitochondrial inner and outer membranes in conjunction with the translocase and the acyl-CoA synthetase, thus combining all four enzymes into a functional unit.

Introduction

The earliest known and best-defined role for carnitine is its requirement for the mitochondrial oxidation of long-chain fatty acids [1]. Fatty acids are rapidly converted into acyl-CoA esters after entering cells, and acyl-CoA esters are oxidized in the mitochondrial matrix for the production of ATP; however, production of acyl-CoA occurs mostly in the cytoplasmic compartment of cells, so that transport across the two mitochondrial membranes is necessary before fatty acids can be oxidized. Mitochondria contain four proteins that act sequentially to accomplish the task of fatty acid activation and transfer into the matrix: (i) acyl-CoA synthetase on the mitochondrial outer membrane which utilizes ATP, coenzyme and fatty acid to produce an fatty acyl-CoA ester, (ii) CPT1 (carnitine palmitoyltransferase I), also located in the mitochondrial outer membrane, which transfers the fatty acyl groups of cytoplasmic acyl-CoA to carnitine to form acylcarnitine; (iii) carnitine–acylcarnitine translocase, located in the mitochondrial inner membrane, which transports acylcarnitine across the inner membrane in exchange for carnitine; and (iv) CPT2 (carnitine palmitoyltransferase II), which transfers fatty acyl groups from acylcarnitine to mitochondrial CoA for oxidation, releasing free carnitine.

CPT1 is recognized as the most important step for regulating mitochondrial fatty acid oxidation [2]. Inhibition of fatty acid oxidation by malonyl-CoA has been thoroughly examined and found to be highly regulated in many different physiological and pathophysiological states. Following the initial discovery that this intermediate in the fatty acid biosynthetic pathway was a potent inhibitor of CPT1 [3], it was soon discovered that fasting dramatically reduced inhibition of CPT1 by malonyl-CoA and released its control of the fatty acid oxidation pathway [4]. CPT1 is not the rate-limiting step for hepatic fatty acid oxidation in the absence of malonyl-CoA. Malonyl-CoA can control fatty acid oxidation only in the normal fed state where insulin sensitizes CPT1 to this inhibitor [5]. In untreated insulin-dependent diabetes (Type 1), ketoacidosis results from the loss of control of CPT1 owing to diminished sensitivity to malonyl-CoA and increased CPT1 activity.

In addition to insulin, thyroid hormone also plays a major role in the regulation of hepatic fatty acid oxidation. The activity of hepatic CPT1 is elevated in hyperthyroidism and the enzyme is slightly less sensitive to malonyl-CoA inhibition [6]. In hypothyroidism, hepatic CPT1 activity is dramatically diminished and its sensitivity to malonyl-CoA is greatly elevated, but hypothyroidism has little or no effect on extrahepatic CPT1 [7].

CPT isoforms

The cloning of cDNAs encoding rat mitochondrial CPT1 [8] and CPT2 [9] shows that these enzymes are distinct proteins, synthesized from separate mRNAs. CPT1 occurs in three isoforms [10,11]: CPT1a (also known as the liver

Key words: acyl-CoA, cardiolipin (diphosphatidylglycerol), carnitine palmitoyltransferase, phospholipid, recombinant carnitine palmitoyltransferase II (recombinant CPT2).

Abbreviations used: CPT1, carnitine palmitoyltransferase I; CPT2, carnitine palmitoyltransferase II.

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or L-isoform) is prevalent in cell types that are involved in glucose-sensing such as pancreatic β-cells; CPT1b was first identified in skeletal and cardiac muscle mitochondria (M-CPT1), but also occurs in brown adipose tissue which has a high capacity for fatty acid oxidation; and CPT1c, which may be a brain-specific protein [12,13] and is reported to be involved in the regulation of body mass [14,15]. CPT1a and CPT1b have a high degree of sequence similarity, but have very important kinetic differences especially under different physiological and pathophysiological states. For example, the sensitivity of CPT1a to inhibition by malonyl-CoA changes by orders of magnitude during fasting and in diabetes [16].

Recombinant CPT2 production and its CPT activity

The production of rat liver CPT2 from bacterial cultures was accomplished by PCR amplification of the open reading frame of pBKS-CPT II.4 (a gift from the late Dr J.D. McGarry) to obtain a cDNA which was inserted into the pFLAG-MAC vector and expressed (Figure 1A). Transformed *Escherichia coli* M15 cells were selected in ampicillin-containing medium, and CPT2 was expressed following induction for 2 h with IPTG (isopropyl β-D-thiogalactopyranoside). Recombinant CPT2 was obtained in a soluble form from bacterial cultures after freezing and thawing and centrifugation at 100 000 ***g***. No CPT activity was detectable in *E. coli* cultures containing the vector without the *CPT2* cDNA insert (Figure 1B); the supernatant was therefore used without further purification. When the recombinant CPT2 was expressed in *E. coli*, it was found to have an acyl-CoA chain-length-specificity identical with that of the enzyme from purified rat liver mitochondrial inner membranes. In both cases, the highest activity was found with decanoyl-CoA (C_{10}), but broad specificity for acyl-CoA was found to extend from C_{10} to C_{18} (Figure 1C). The K_m values for carnitine for both the mitochondrial preparation and the recombinant enzyme were identical.

Effect of membrane microenvironment on CPT

As the primary regulatory enzyme in the fatty acid oxidation pathway, CPT1 is characterized by many levels of control, including substrate concentration, specific inhibition by malonyl-CoA, changes in sensitivity to inhibition by malonyl-CoA and hormonal regulation of the expression of the CPT1 gene. In the present article, we provide additional data on the specific effects of phospholipids on CPT1 of the hepatic isolated outer membranes and on CPT2 after expression in *E. coli*.

The interaction of CPT1 with membrane phospholipids was first proposed by Kolodziej and Zammit in 1990 [17] because of a large temperature effect on malonyl-CoA inhibition. The addition of phospholipids directly to the assay medium without making certain that phospholipid vesicles had first been formed results in specific effects of

Figure 1 | Separation of *E. coli* proteins by preparative isoelectric focusing, expression of CPT2 in *E. coli*, and its acyl-CoA chain-length-specificity

CPT2 activity was found to band on isoelectric focusing (Rotofor) to the appropriate isoelectric point (pH 6.2) based on the calculated pI of the CPT2 construct (**A**). CPT activity was found in lysates of *E. coli* that contained the expression plasmid with the CPT2 insert (**B**). CPT2 from *E. coli* possessed the same acyl-CoA chain-length-specificity as that known for the purified rat liver enzyme, i.e. highest activity with C_{10} to C_{18} (**C**).

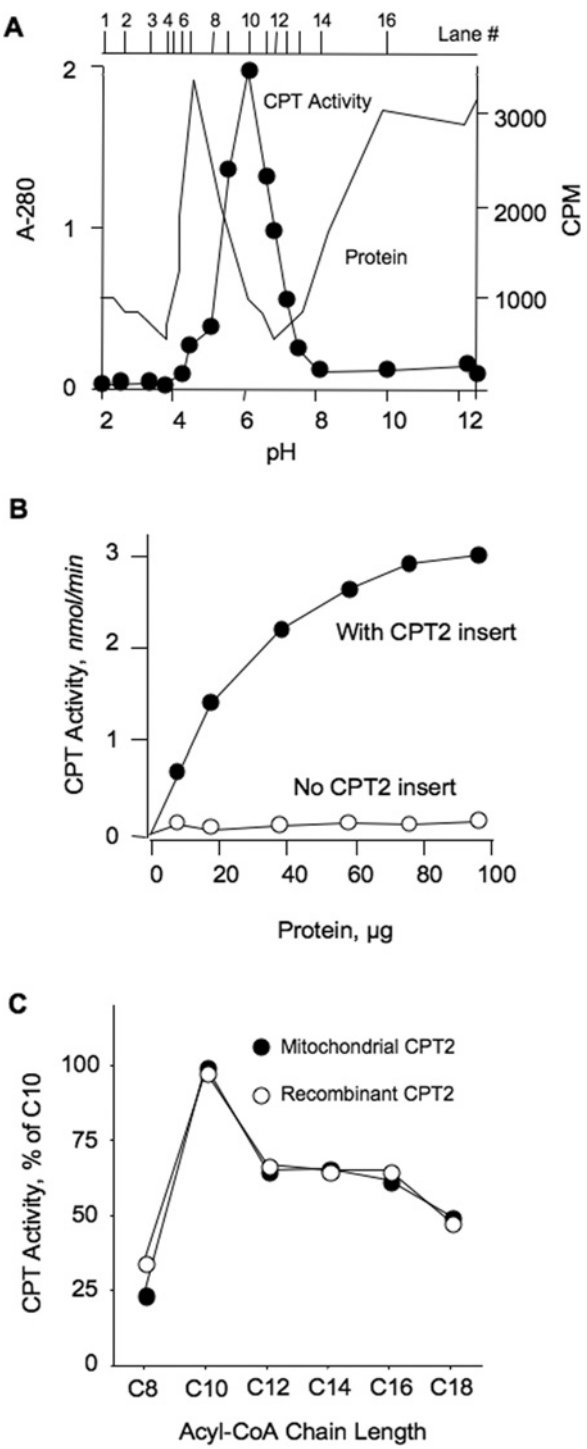
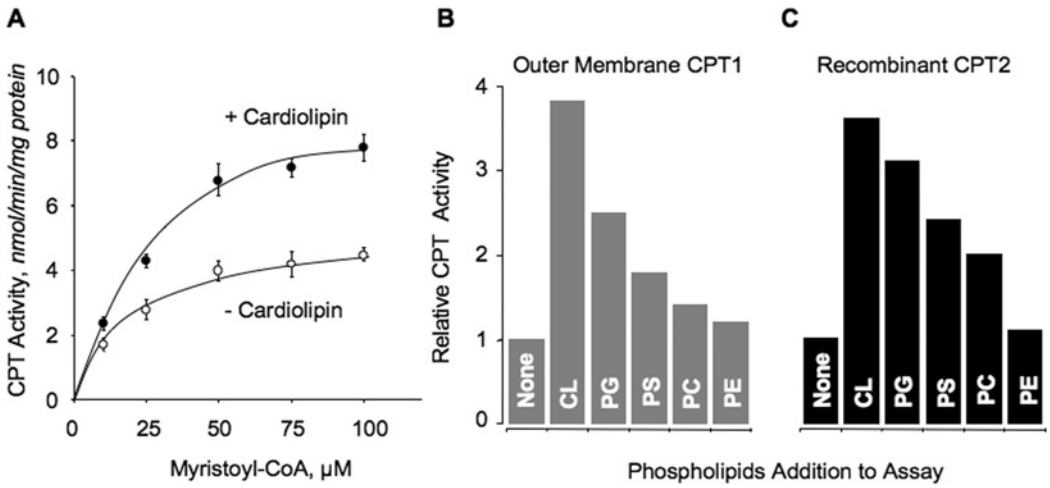


Figure 2 | Phospholipid activation of CPT1 and recombinant CPT2

Cardiolipin increased CPT1 activity of intact mitochondria, at the highest concentration of myristoyl-CoA used; the increased activity was almost 2-fold (**A**). Several phospholipids, especially cardiolipin and phosphatidylglycerol caused significant activation of CPT1 activity of isolated mitochondrial outer membranes (**B**). When the recombinant CPT2 was subjected to the same treatment conditions, several phospholipids also had large activating effects (**C**). CL, cardiolipin; PG, phosphatidylglycerol; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine.



some phospholipids on malonyl-CoA inhibition of CPT1, with little effect on CPT1 activity [18]. Arrhenius plots have indicated that both CPT1 activity and malonyl-CoA inhibition are characterized by a transition temperature which occurs at the temperature at which membrane phospholipids convert from a liquid into a liquid-crystalline structure [19]. These experiments indicate that CPT1 is very closely associated with the membrane and greatly affected by its composition.

Phospholipids increase CPT activity

Addition of cardiolipin (diphosphatidylglycerol) to intact liver mitochondria increases CPT1 activity (Figure 2A) as reported previously [18]. We have also shown that the sensitivity of CPT1 from intact liver mitochondria to inhibition by malonyl-CoA is greatly affected by addition of cardiolipin to the assay system [18]. Table 1 shows that cardiolipin has no effect on the apparent K_m for acyl-CoA; however, it increase the K_m for carnitine 6-fold. It also increases the activity of CPT1 approximately 4-fold and impressively increases the sensitivity of the enzyme to inhibition by malonyl-CoA approximately 60-fold. When several phospholipid micellar preparations were examined for their effects on isolated mitochondrial outer membranes, i.e. CPT1 activity, there was significant activation by several phospholipids, especially cardiolipin and phosphatidylglycerol (Figure 2B).

Rat liver CPT2 is normally associated with inner mitochondrial membranes and has been isolated and purified following removal from membranes using detergents [9,20]. However, we found the recombinant CPT2 in the soluble fraction (100 000 ***g*** supernatant fraction) of bacterial extracts.

Table 1 | Effect of cardiolipin on the kinetic characteristics of hepatic mitochondrial CPT1

Values for K_m were obtained in intact mitochondria from plots of $1/v$ against $1/S$. Values for K_i were estimated from Dixon plots using five concentrations of malonyl-CoA and each of three different substrate concentrations. Values are means ± S.E.M. for three separate preparations of mitochondria. $^*P<0.05$ compared with values obtained in the absence of cardiolipin.

Kinetic constant	Control	Plus cardiolipin
K_m (app) for myristoyl-CoA (μM)	28.2 ± 0.5	27.5 ± 0.6
K_m for carnitine (mM)	0.5 ± 0.2	3.0 ± 1.6*
K_i for malonyl-CoA (μM)	1.5 ± 0.4	0.025 ± 0.005*
V_{max} (nmol/min per mg of protein)	9.1 ± 0.8	41 ± 19*

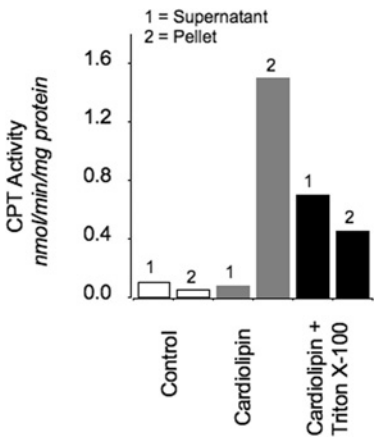
This gave us an opportunity to study the effects of membrane phospholipids on the recombinant enzyme. When the recombinant CPT2 was subjected to the same treatments as those used for isolated mitochondrial outer membranes, several phosphlipids produced large activating effects (Figure 2C). Cardiolipin activated recombinant CPT2 almost 4-fold, whereas the structurally similar phopholipid, phosphatidylglycerol, activated the enzyme 3-fold, and phosphatidylserine and phosphatidylcholine both produced approximately 2-fold activation.

CPT2 is tightly associated with membrane phospholipids

In order to ascertain the extent of association of the recombinant CPT2 with phospholipids, centrifugation

Figure 3 | **Effects of phospholipids on solubility of recombinant CPT2**

Recombinant CPT2 was mainly in the soluble fraction before interaction with cardiolipin. Triton X-100 disrupted the interaction between the recombinant protein and the phospholipid.

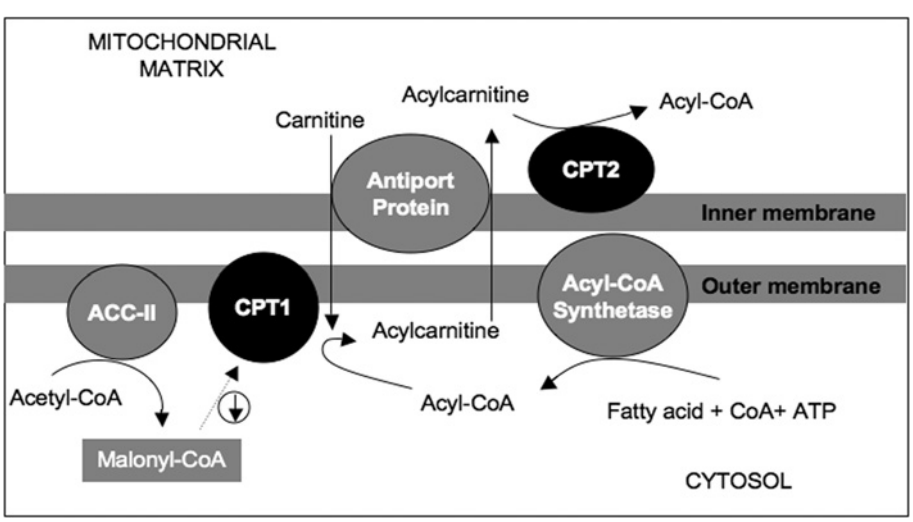


experiments were conducted in which the recombinant enzyme was centrifuged at 100 000 *g* both before incubation with cardiolipin and afterwards. Most of the recombinant CPT2 was found in the soluble fraction before interaction with cardiolipin (Figure 3), but more than 90% of the enzyme was precipitated by centrifugation following a 20 min incubation with cardiolipin, indicating that there was substantial interaction between the recombinant protein and the phospholipid. When the detergent Triton X-100 was added to the phospholipid and enzyme incubations, the interaction between protein and phospholipid was apparently disrupted because, under those conditions, most of the recombinant CPT2 remained in solution during centrifugation (Figure 3).

Model reconciling fatty-acid-oxidizing enzymes and the mitochondrial membranes

Our observation of the activation of the recombinant CPT2 by phospholipids is surprising. It is also interesting that the structurally similar phospholipids cardiolipin and phosphatidylglycerol produced their greatest effects on both CPT1 and CPT2. The precipitation of the previously soluble recombinant CPT2 by cardiolipin suggests that an artificial membrane system was formed. It is not known why cardiolipin was the best activator of these enzymes, but cardiolipin is unique among the phospholipids tested since it possesses two pairs of hydrophobic fatty acid chains that can be inserted into two adjacent membranes at once or can bend to insert all four fatty acids into the same membrane. This characteristic suggests the interesting possibility that it may function normally to hold the fatty-acid-oxidizing enzymes in the active functional conformation between the mitochondrial inner and outer membranes. Since the carnitine–acylcarnitine translocase and the acyl-CoA synthetase are also activated by cardiolipin [21], it seems logical to propose that all four enzymes may be held together in a functional unit (Figure 4). The Figure illustrates how fatty acid is activated outside the outer membrane at the acyl-CoA synthetase active site [22] and how the acyl-CoA can be immediately transferred to the active site of CPT1 which would be accessible to cytosolic malonyl-CoA. It has also been proposed that CPT1 may be located in contact sites where inner and outer mitochondrial membranes are joined [23,24]. This model suggests that there should be a very close interaction of all four enzymes

Figure 4 | **Model of fatty acid activation and transport system for mitochondrial oxidation**



with the possibility that they may exist *in vivo* as a complex.

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References

1 Fritz, I.B. (1963) Carnitine and its role in fatty acid metabolism. Adv. Lipid Res. **1**, 285–334
2 Bieber, L.L. (1988) Carnitine. Annu. Rev. Biochem. **57**, 261–283
3 McGarry, J.D., Mannaerts, G.P. and Foster, D.W. (1977) A possible role for malonyl-CoA in the regulation of hepatic fatty acid oxidation and ketogenesis. J. Clin. Invest. **60**, 265–270
4 Cook, G.A., Otto, D.A. and Cornell, N.W. (1980) Differential inhibition of ketogenesis by malonyl-CoA in mitochondria from fed and starved rats. Biochem. J. **192**, 955–958
5 Cook, G.A. and Gamble, M.S. (1987) Regulation of carnitine palmitoyltransferase by insulin results in decreased activity and decreased apparent K_i values for malonyl-CoA. J. Biol. Chem. **262**, 2050–2055
6 Stakkestad, J.A. and Bremer, J. (1983) The outer carnitine palmitoyltransferase and regulation of fatty acid metabolism in rat liver in different thyroid states. Biochim. Biophys. Acta **750**, 244–252
7 Saggerson, E.D. and Carpenter, C.A. (1986) Carnitine palmitoyltransferase in liver and five extrahepatic tissues in the rat: inhibition by DL-2-bromopalmitoyl-CoA and effect of hypothyroidism. Biochem. J. **236**, 137–141
8 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
9 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
10 McGarry, J.D. and Brown, N.F. (1997) The mitochondrial carnitine palmitoyltransferase system: from concept to molecular analysis. Eur. J. Biochem. **244**, 1–14
11 Zammit, V.A. (2008) Carnitine palmitoyltransferase 1: central to cell function. IUBMB Life **60**, 347–354
12 Price, N., Van Der Leij, F., Jackson, V., Corstorphine, C., Thomson, R., Sorensen, A. and Zammit, V. (2002) A novel brain-expressed protein related to carnitine palmitoyltransferase I. Genomics **80**, 433–442
13 Sierra, A.Y., Gratacos, E., Carrasco, P., Clotet, J., Urena, J., Serra, D., Asins, G., Hegardt, F.G. and Casals, N. (2008) CPT1c is localized in endoplasmic reticulum of neurons and has carnitine palmitoyltransferase activity. J. Biol. Chem. **283**, 6878–6885
14 Wolfgang, M.J., Kurama, T., Dai, Y., Suwa, A., Asaumi, M., Matsumoto, S., Cha, S.H., Shimokawa, T. and Lane, M.D. (2006) The brain-specific carnitine palmitoyltransferase-1c regulates energy homeostasis. Proc. Natl. Acad. Sci. U.S.A. **103**, 7282–7287
15 Dai, Y., Wolfgang, M.J., Cha, S.H. and Lane, M.D. (2007) Localization and effect of ectopic expression of CPT1c in CNS feeding centers. Biochem. Biophys. Res. Commun. **359**, 469–474
16 Park, E.A. and Cook, G.A. (1998) Differential regulation in the heart of mitochondrial carnitine palmitoyltransferase-I muscle and liver isoforms. Mol. Cell. Biochem. **180**, 27–32
17 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
18 Mynatt, R.L., Greenhaw, J.J. and Cook, G.A. (1994) Cholate extracts of mitochondrial outer membranes increase inhibition by malonyl-CoA of carnitine palmitoyltransferase-I by a mechanism involving phospholipids. Biochem. J. **299**, 761–767
19 Kashfi, K. and Cook, G.A. (1995) Temperature effects on malonyl-CoA inhibition of carnitine palmitoyltransferase IBiochim. Biophys. Acta **1257**, 133–139
20 Miyazawa, S., Ozasa, H., Furuta, S., Osumi, T. and Hashimoto, T. (1983) Purification and properties of carnitine acetyltransferase from rat liver. J. Biochem. (Tokyo) **93**, 439–451
21 Noel, H. and Pande, S.V. (1986) An essential requirement of cardiolipin for mitochondrial carnitine acylcarnitine translocase activity: lipid requirement of carnitine acylcarnitine translocase. Eur. J. Biochem. **155**, 99–102
22 Pande, S.V. and Blanchaer, M.C. (1970) Preferential loss of ATP-dependent long-chain fatty acid activating enzyme in mitochondria prepared using Nagarse. Biochim. Biophys. Acta **202**, 43–48
23 Hoppel, C. (1992) Carnitine palmitoyltranferases. In Current Concepts in Carnitine Research (Carter, A.L., ed.), pp. 153–164, CRC Press, Boca Raton
24 Fraser, F., Padovese, R. and Zammit, V.A. (2001) Distinct kinetics of carnitine palmitoyltransferase i in contact sites and outer membranes of rat liver mitochondria. J. Biol. Chem. **276**, 20182–20185

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