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Partial characterization of a malonyl-CoA-sensitive carnitine O-palmitoyltransferase I from *Macrobrachium borellii* (Crustacea: Palaemonidae)

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ABSTRACT

The shuttle system that mediates the transport of fatty acids across the mitochondrial membrane in invertebrates has received little attention. Carnitine O-palmitoyltransferase I (EC 2.3.1.21; CPT I) is a key component of this system that in vertebrates controls long-chain fatty acid β-oxidation. To gain knowledge on the acyltransferases in aquatic arthropods, physical, kinetic, regulatory and immunological properties of CPT of the midgut gland mitochondria of *Macrobrachium borellii* were assayed. CPT I optimum conditions were 34 °C and pH=8.0. Kinetic analysis revealed a *K*m for carnitine of 2180±281 μM and a *K*m for palmitoyl-CoA of 98.9±8.9 μM, while V_{max} were 56.5±6.6 and 36.7±4.8 nmol min^{-1} mg $protein^{-1}$, respectively. A Hill coefficient, $n \sim 1$, indicate a Michaelis–Menten behavior. The CPT I activity was sensitive to regulation by malonyl-CoA, with an IC_{50} of 25.2 μM. Electrophoretic and immunological analyses showed that a 66 kDa protein with an isoelectric point of 5.1 cross-reacted with both rat liver and muscle-liver anti CPT I polyclonal antibodies, suggesting antigenic similarity with the rat enzymes. Although CPT I displayed kinetic differences with insect and vertebrates, prawn showed a high capacity for energy generation through β-oxidation of long-chain fatty acids.

1. Introduction

To obtain energy from lipid reserves animals oxidize fatty acids inside mitochondria. To get into this organelle, long-chain fatty acids are first activated on the mitochondrial outer membrane by long-chain acyl CoA synthetase, but as the inner membrane is not permeable to acyl-CoA, they must be transported by a shuttle mechanism. The transport system consists of three proteins whose first component is carnitine acyltransferase I (CAT I), an integral protein of the outer mitochondrial membrane. Carnitine O-palmitoyl-transferase I (CPT I; EC 2.3.1.21) is a member of this protein family that catalyses the transfer of long fatty acyl groups from cytosolic fatty acyl-CoA to carnitine (Schomburg et al., 2006). The acyl-carnitine is then translocated into the mitochondrial matrix in an exchange reaction catalyzed by carnitine: acyl-carnitine translocase, an integral inner membrane protein. Within the matrix the acyl-carnitines are then reconverted into the respective acyl-CoAs by CAT II, an enzyme associated with the inner leaflet of the mitochondrial inner membrane (Kerner and Hoppel, 2000). In vertebrates, CPT I has a key function controlling the flux through β-oxidation by virtue of its sensitivity to inhibition by malonyl-CoA (Mills et al., 1983).

The malonyl-CoA is a reversible inhibitor of CPT I produced during the first step of fatty acid biosynthesis. In vertebrates it provides a simple mechanism for reciprocal control of two opposite pathways, fatty acid synthesis and fatty acid oxidation. Inhibition of CPT I by malonyl-CoA plays therefore a major role in vertebrate physiological regulation of fatty acid oxidation according to prevailing hypotheses concerning the control of mitochondrial β-oxidation flux, though a sharing control with other enzymes has also been proposed (Eaton, 2002).

In a previous work, we studied the capacity for energy generation through the activation and β-oxidation of palmitic acid in the midgut gland mitochondria of the freshwater prawn *M. borellii* (Lavarías et al., 2006). This prawn has an active lipid metabolism in this organ, which has the combined functions of the mammalian liver, pancreas and adipose tissue (Irazú et al., 1992; González Baró and Pollero, 1993, 2000). CPT I has perhaps only been studied in great detail in vertebrates, particularly in rats and humans, and there are much fewer reports available on this shuttle system in fish and other chordates (Crabtree and Newsholme, 1972; Gutieres et al., 2003; Morash et al., 2008). There is no information on CPT characterization in crustaceans. In fact, to our knowledge, the information of CPT I characteristics from invertebrates is limited to a partial characterization of a CPT in the grasshopper *Valanga nigricornis* (Lee and Tan, 1983) and an *in vitro* study of *Drosophila* CPT expressed in yeast cultures (Jackson et al., 1999).

Considering that *M. borellii* midgut gland is the major site of lipid metabolism and that information on several lipid catabolic enzymes is

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available (Irazú et al., 1992; González Baró and Pollero, 1993; Lavarías et al., 2006, 2007), we selected it as a model organ for the study of crustacean mitochondrial CPT I activity.

To increase our knowledge about crustacean lipid metabolism, we report here some physical, kinetic, and immunological properties of CPT from the midgut gland mitochondria of a crustacean. In addition, CPT I regulatory properties of malonyl-CoA was also investigated.

2. Materials and methods

2.1. Chemicals

Palmitoyl-CoA, L-carnitine, malonyl-CoA and a proteinase inhibitor cocktail were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of the highest grade commercially available.

2.2. Sample collection

Adults of *M. borellii* were sampled in an uncontaminated watercourse close to the La Plata river, Argentina (20 Km to the SW from La Plata) (Lavarías et al., 2005). They were taken to the laboratory and kept in dechlorinated running water at 22±2 °C, 14:10 h L: D photoperiod for at least a week for acclimation (Collins and Petriella, 1999). The animals were starved for 48 h before experiments, a standard practice of our laboratory.

2.3. Preparation of mitochondrial fraction

The midgut glands from several individuals were pooled, weighed and chilled on ice in the buffer. They were then homogenized in 0.25 M sucrose containing 2 mM EDTA, 0.1 mM DTT and buffered with 10 mM HEPES pH 7.4 and a protease inhibitor cocktail (Sigma), using a glass-Teflon potter homogenizer (Thomas, Philadelphia, PA, USA). The crude homogenate was filtered and centrifuged in a Sorvall RC-2 (Newtown, CT, USA) at 600 g at 4 °C for 10 min to remove cellular debris. The supernatant was filtered and centrifuged at 10,000 *g* for 20 min to obtain the mitochondrial fraction. Total protein was determined by the method of Bradford (1976).

2.4. Carnitine palmitoyltransferase assay

Before starting the kinetic assays, an experiment to find the optimal values for protein concentration, incubation time, pH, and temperature was performed (see Results). The procedure used was a modification of that applied by Bieber and Fiol (1986). Enzyme activity was measured in the forward direction (formation of palmitoylcarnitine) by monitoring the initial rate of CoASH release with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) at 412 nm. The reaction mixture, in a total volume of 0.3 mL, contained 65 mM HEPES buffer, 0.13% Triton X-100, 1.4 mM EDTA, with varying amounts of L-carnitine and palmitoyl-CoA. The reaction was started by the addition of mitochondria. Hydrolase activity (blank) was calculated by measuring the carnitine-independent release of CoASH and subtracted. Carnitine was assayed in the range 0.4–2.6 mM and palmitoyl-CoA in the range 0.01–1.2 mM.

CPT I activity as a function of temperature was obtained using 50 μg mL^{-1} protein, 30 mM carnitine and 1 mM palmitoyl CoA. CPT I activity as a function of protein concentration was recorded between 0–300 μg protein mL^{-1}, at 30 mM carnitine, 1 mM palmitoyl CoA, and 34 °C.

Assuming that, as in vertebrates, only CPT I isoform is sensitive to malonyl-CoA inhibition (McGarry et al., 1983), activity values were recorded at increasing malonyl-CoA concentrations until minimum activity was obtained. Residual activity was ascribed to CPT II isoform and was subtracted in calculations of CPT I activity.

2.5. Inhibition of CPT I by malonyl-CoA

In order to evaluate the inhibition of CPT I activity by malonyl CoA, different concentrations of the inhibitor were pre-incubated with mitochondrial preparations for 10 min (Zammit, 1983a). The reaction was started by the addition of pamitoyl-CoA 1 mM in the test tubes and followed spectrophotometrically as described above.

2.6. Cross-reactivity between rat and prawn anti-CPT I antibodies

To immunodetect CPT I in *M. borellii* midgut gland mitochondrial fraction, 2 rabbit polyclonal anti-sera kindly provided by Dr. Hoppel's laboratory were used. One anti-sera was raised against a specific oligopeptide for rat liver CPT I isoform and the other one against an oligopeptide common to both, liver and muscle CPT I isoforms (Hoppel et al., 2001).

Proteins of midgut gland mitochondria were separated by electrophoresis (12% SDS-PAGE) and electroblotted onto nitrocellulose membranes (Amersham) in a Mini Trans-blot Cell (Bio Rad Laboratories), using 25 mM Tris–HCl, 192 mM glycine, 20% (v/v) methanol, pH=8.3 buffer. After blocking for 2 h at 37 °C with 5% (w/v) non-fat dry milk in PBS-Tween, the membranes were incubated overnight at 4 °C with both anti-sera diluted 1/1000 in 3% (w/v) non fat dry milk in PBS-Tween. Specific antigens were detected by goat anti-rabbit IgG horseradish peroxidase conjugate (Bio Rad) diluted 1/2500 in 3% (w/v) non fat dry milk in PBS-Tween. Immunoreactivity was visualized by electro-chemiluminiscence.

2.7. Isoelectric point determination

Two-dimensional electrophoresis was carried out with IPGs-Isoelectric Focusing (IEF) in the first dimension and SDS-PAGE in the second dimension (Gorg et al., 1988). The IEF was performed using an Ettan IPGphor III (GE Healthcare, Uppsala, Sweden) and 7 cm linear pH 4–7 immobiline dry strips (GE Healthcare). Rehydration and loading of the strip was carried out overnight at room temperature in a dilution buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% v/v IPG Buffer 4–7 linear (GE Healthcare)) containing 120 μg protein. After IEF, the immobiline dry strips were equilibrated at room temperature for 20 min in a buffer containing: 75 mM Tris–HCl, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 0.002% w/v bromophenol blue and 1% w/v DTT, and then alkylated for 20 min in the above buffer, but with 4.5% w/v iodacetamide in place of DTT. For the SDS-PAGE second-dimension, the IPG strips were sealed on top of 1.5 mm thick 12% polyacrylamide gels. Vertical electrophoresis was carried out at 15 mA/gel. Immunoblot analysis was performed as described above.

2.8. Statistical analyses

Data were analysed either by Student's *t*-test or analysis of variance using Instat v.2.0. Results were considered significant at 5% level.

3. Results

3.1. Kinetic characterization of prawn carnitine palmitoyltransferase I

The palmitoylcarnitine synthesis as a function of protein concentration is depicted in Fig. 1A. Considering the enzyme activity has a linear response at least up to 67 μg/mL of mitochondrial protein, assays were performed using 50 μg/mL of mitochondrial protein. Optimal temperature for CPT activity was found to be 34 °C (specific activity (SA) 25.6 nmol min^{-1} mg $protein^{-1}$). CPT activity decayed at higher temperatures as is usual in thermo labile enzymes (Fig. 1B). The activity of CPT was assayed as a function of pH and pH 8

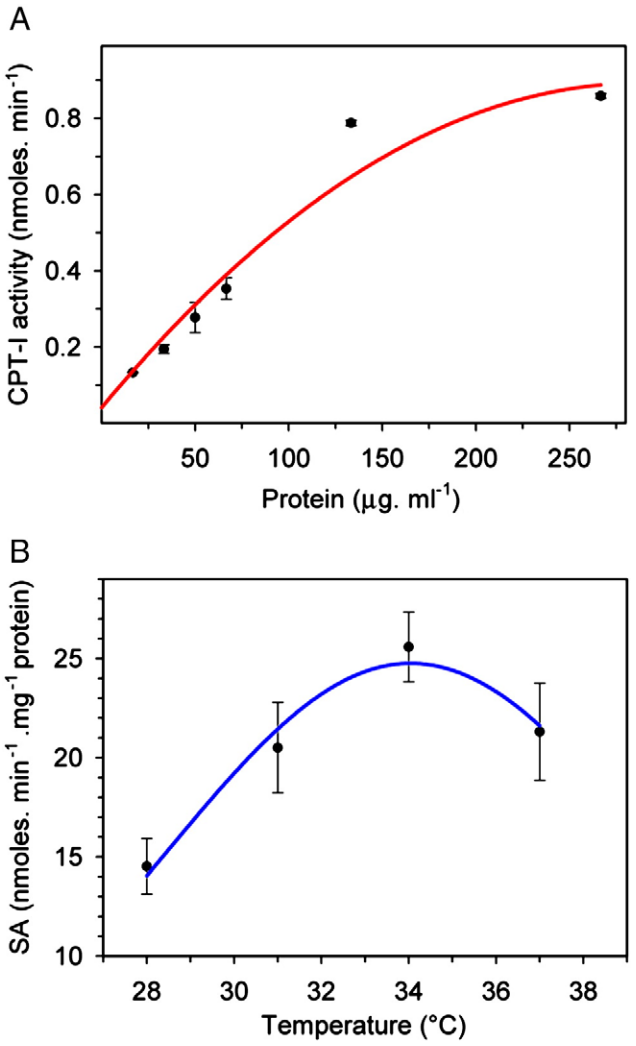

Fig. 1. Effect of protein concentration (A) and temperature (B) on the activity of mitochondrial CPT I in midgut gland of *M. borellii*. SA: Specific activity. Values represent the mean ± one SD, $n=3$.

was selected for the assays (results not shown). The rate of product formation decreased along the incubation time, so assays were always performed in the 1–2.5 min time window.

Using the conditions optimized as above described, the synthesis of palmitoylcarnitine was then assayed as a function of both substrates: palmitoyl-CoA and L-carnitine. The reaction followed a normal Michaelis–Menten kinetics in the substrate concentration range analyzed as shown in the Lineweaver–Burk plots (Fig. 2). The Hill coefficient value for both substrates was near 1. The apparent Michaelis–Menten constant (*K*m) and V_{max} for palmitoyl-CoA were 98.9±8.9 µmol l^{-1}, and 36.7±4.8 nmol min^{-1} mg $protein^{-1}$ respectively. For the bioassays we selected a substrate concentration of 1 mM palmitoyl-CoA. When CPT I activity was studied as a function of L-carnitine substrate, the kinetic parameters *K*m and V_{max} were 2.18± 0.28 mM, and 56.5±6.6 nmol min^{-1} mg $protein^{-1}$, respectively. The concentration of L-carnitine chosen for further experiments was 30 mM.

3.2. Inhibition of CPT by malonyl-CoA

The CPT I activity of prawn was malonyl-CoA sensitive (Fig. 3), since only ≈6.6% of CPT activity still remained after the addition of 600 µM malonyl-CoA (assuming that only prawn CPT I is sensitive to CoA esters (McGarry et al., 1983).

The concentration of malonyl-CoA required to inhibit 50% of the malonyl-CoA sensitive activity (IC_{50}) of CPT I is depicted in Fig. 3. In a preliminary experiment, a comparison was made between the effect of adding (1) different malonyl-CoA concentrations and a fixed concentration of palmitoyl-CoA to the reaction mix at the same time, starting the reaction by the addition of mitochondria, or (2) pre-incubating the inhibitor with mitochondria for 10 min and then starting the reaction by the addition of the substrate. The results showed that the IC_{50} was 105.8 µM without pre-incubation (not shown) and 25.2 µM with 10 min pre-incubation of the enzyme with malonyl-CoA, indicating a sensitivity 4 times higher for the latter condition (Fig. 3).

3.3. Physical and immunological characterization of CPT I

Cross-reaction of polyclonal antibodies raised against oligopeptides of rat muscle/liver and rat liver CPT I with mitochondrial proteins from the midgut gland of *M. borellii* are shown in Fig. 4. Both antibodies cross-reacted with a single protein band under dissociating conditions, a remarkable result considering that the tested

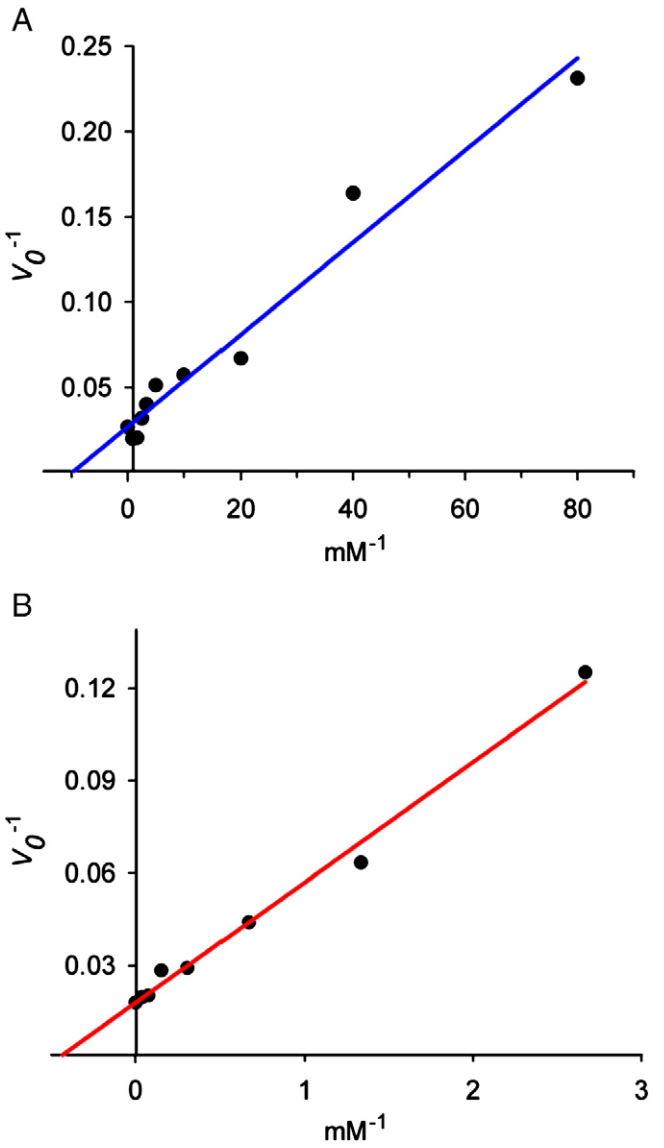

Fig. 2. Kinetic analysis of *Macrobrachium borellii* CPT I. Lineweaver–Burk plots of the activity of CPT I with respect to its substrates palmitoyl-CoA (A) and carnitine (B). *Vo*: Initial velocity (nmol L^{-1} min^{-1} mg $protein^{-1}$). Values represent the mean of three determinations. (A) $R^2=0.96$; (B) $R^2=0.99$.

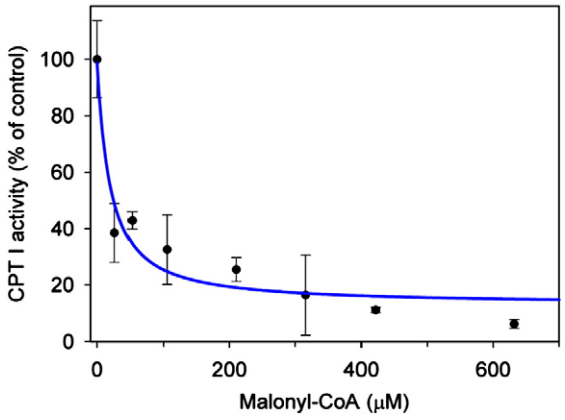

Fig. 3. Inhibition of *Macrobrachium borellii* midgut gland CPT activity by malonyl-CoA. Values are the mean of three experiments.

mitochondria belong to an invertebrate. This protein has an apparent molecular mass of 66 kDa and an isoelectric point of 5.1.

4. Discussion

Early studies on the assimilation of nutrients in crustaceans were limited to morphological and histological descriptions of their complex digestive systems, until more recently, when biochemistry and molecular biology studies have enlarged our knowledge about the processes of digestion and food utilization in these arthropods. In particular, crustacean midgut gland has been recognized as possessing a very active lipid metabolism. Our laboratory has previously characterized several key enzymes of the catabolic pathways such as a soluble triacylglycerol lipase, mitochondrial palmitoyl-CoA synthetase (ACS) and the β-oxidation system, as well as some of their anabolic pathways including microsomal ACS kinetics and glycerolipid synthesis, largely unknown in these arthropods (González Baró et al., 1990; Irazú et al., 1992; Lavarías et al., 2006, 2007). In the present paper we have studied some characteristics and potential control of mitochondrial fatty acid channeling for β-oxidation in crustaceans.

4.1. Kinetic comparisons between the CPT I activities of prawn with those of insect and vertebrates

The kinetics of *Macrobrachium* midgut gland CPT I activity with respect to its substrates carnitine and palmitoyl-CoA was determined in mitochondrial fraction. Saturation plots of CPT I yielded a hyperbolic behaviour, similar to what was obtained in rat and fish using similar protocols (Richards et al., 1991; Stonell et al., 1997). CPT I *K*m for carnitine was higher than those reported for insect and mammalian isoforms whereas that for palmitoyl-CoA was in the same order as those of *Drosophila* and higher than mammalian isoforms (Table 1) (McGarry et al., 1983; Mills et al., 1984; Weis et al., 1994; Jackson et al., 1999). As in other eukaryotes, carnitine was found to be essential for crustacean fatty acid β-oxidation (Kallapur et al., 1983). Although the affinity toward both substrates was lower than that of vertebrates, the corresponding V_{max} in prawn CPT was decidedly higher than mammals and fish (Table 1). In particular, rainbow trout maximal catalytic capacity of CPT I. was 0.53–0.25 nmol min^{-1} mg^{-1} wet wt. tissue (measured by a spectrophotometric method in crude homogenates) (Egginton, 1996), and similar results were found by other authors in salmon (Froyland et al., 1998; Gutieres et al., 2003). If we consider that control of β-oxidation flux in prawn is largely at the level of entry of acyl groups to mitochondria, CPT I V_{max} values indicates that crustacean midgut gland has a high capacity for energy generation, at least using saturated long-chain fatty acids. This is in agreement with previous studies concerning the utilization of lipids in *M. borellii* midgut gland that showed high mitochondrial oxidation of palmitic acid, a major fatty acid in midgut gland as well as in the diet of this species (Pollero et al., 1991; Irazú et al., 1992).

4.2. Inhibition by malonyl-CoA

In mammals, high concentrations of malonyl-CoA accompany high rates of fatty acid synthesis and simultaneously inhibit the first step required to admit long-chain acyl moieties to β-oxidation within the mitochondrial matrix. This reciprocal effect is amplified through changes in the sensitivity of CPT I toward malonyl-CoA. In this regard, prawn seems to use a similar strategy as midgut gland mitochondria preincubation with malonyl-CoA resulted in 4-times increase in the sensitivity of CPT I activity to malonyl-CoA compared with that shown by the enzyme not preincubated. In mammals, increased hepatic content of malonyl-CoA, renders the enzyme more sensitive to malonyl-CoA inhibition (Zammit, 1983b, 1999). The enzyme is about 8 times less sensitive than rat liver CPT I and much less sensitive than *Drosophila,* muscle rat or trout CPT I (Table 1). It worth noting that the difference in the sensitivity of CPT I to malonyl-CoA inhibition between prawn midgut and rat liver was thin compared to the situation between muscle and liver in rats where the IC50 differs about 100 times.

Besides, the sensitivity of CPT I towards malonyl-CoA could have also been lowered by the 48 h-starvation before experiments. In this regard, it has been reported that the inhibitory effect of malonyl-CoA in liver mitochondria is diminished several times after rat starvation (Zammit et al., 1998). In fact, IC_{50} value of CPT I towards malonyl-CoA is of the same order as that reported for 48 h-starved rat (Schomburg et al., 2006). The existence of a malonyl-CoA-sensitive CPT I in prawn, insect and chordates points to a widespread distribution of this mechanism of control of fatty acid oxidation and other cellular processes. It is probable that, as in mammals, CPT I is subject to a complex regulation that includes modulation of the transcriptional levels, malonyl-CoA concentration, changes in the enzyme sensitivity to malonyl CoA as well as additional mechanisms of short term control of CPT activity such as location in the mitochondria and close interaction with ACS (Kerner and Hoppel, 2000).

4.3. Physical and immunological characterization

The 66 kDa mitochondrial protein that cross-reacted with rat CPT I is in the same MW order of vertebrate CPT I, that ranges from 63 to 88 kDa (Jackson et al., 1999) though the pI is lower than the theoretical value calculated for rat L and M-CPT I. The fact that it was recognized by two anti-peptide antibodies raised against different regions of the

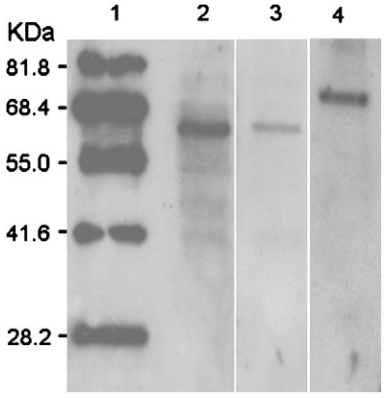

Fig. 4. Immunodetection after SDS-PAGE of CPT I in midgut gland mitochondrial fraction of *M. borellii* using anti (rat CPT I) antibodies. Lane 1: Molecular weight markers, lane 2: Midgut gland immunoreactive proteins to antiserum against rat liver-muscle CPT I isoform (diluted 1/1000); Lane 3: Midgut gland immunoreactive proteins to antiserum against rat liver CPT I isoform (diluted 1/1000). Lane 4: Rat liver mitochondria (positive control).

Table 1
CPT I activity from *Macrobrachium* and other organisms

Organism	Apparent carnitine *K*m (μM)	Apparent palmitoyl-CoA *K*m (μM)	Apparent V_{max} (carnitine) nmol min^{-1} mg $protein^{-1}$	Apparent V_{max} (palmitoyl-CoA) nmol min^{-1} mg $protein^{-1}$	IC_{50}malonyl-CoA (μM)
Arthropoda: Crustacea *Macrobrachium borellii*(1)	2.180	100	56	37	25.2
Arthropoda: Insecta *Drosophila melanogaster*(2)	406	105	ND	ND	0.74
Arthropoda: Insecta *Valanga nigricornis* (3)	ND	39	ND	22	ND
Vertebrata: Mammal *Rattus norvegicus* L-CPT I (4)	120	65	7		3.0
Vertebrata: Mammal *Rattus norvegicus* M-CPT I (4)	744	34	14		0.03
Vertebrata: Mammal *Rattus* H *norvegicus* -CPT (5)	197	35	10	8.4	0.16
Vertebrata: Aves *Gallus gallus* (embryo) (6)	140	32	ND	7	ND
Vertebrata: Pisces *Geotria australis*(7, 8)	ND	7.8	ND	4.5	0.12*
Vertebrata: Pisces *Oncorhynchus mykiss*(9)	ND	ND	ND	0.17–0.53*	0.11–0.43*

1:present paper; 2: Jackson et al. (1999); 3: Lee and Tan (1983); 4: McGarry et al. (1983), Mills et al. (1984); 5: Weis et al. (1994); 6: Schomburg et al. (2006); 7: Stonell et al. (1997); 8: Cake et al. (1995); 9: Egginton (1996), Froyland et al. (1998), Gutieres et al. (2003).
ND: Not Determined.
*Values expressed by mg total homogenate protein.

primary sequence deduced for rat mitochondrial M- and L-CPT I indicates that prawn CPT presents similar epitopes. Data suggest that the catalytic protein is composed of a single polypeptide but the presence of a regulatory subunit binding malonyl-CoA cannot be discarded in this prawn. In addition to immunological identification of proteins, determining enzymatic activity, such as the malonyl-CoA sensitivity, is important from a functional standpoint. Thus, the CPT I inhibition by malonyl CoA together with the fact that an antibody anti CPT I from mammals recognize a protein in the mitochondrial fraction are strong indications of a functional and structural homology between CPT I from rat and mitochondrial CPT from *M. borellii.* This contrasts with *Drosophila*, where none of the anti-(rat CPT I) antibodies tested recognized CPT I expressed in yeast on Western blots (Lavarías et al., 2007). In view of the relatively low similarity between the sequence of *Drosophila* CPT I and that of mammalian CPT I, further studies of the crustaceans CPT such as the cloning of the gene are needed to provide new insights for the elucidation of structure-function relationships in this key enzyme.

4.4. Physiological considerations

Three main observations have emerged from the present study: first, that prawn midgut gland has a malonyl-CoA-sensitive CPT I, secondly, that CPT I properties may allow a high capacity for midgut gland mitochondria to generate energy through β-oxidation of its long chain fatty acids, and thirdly, that *Macrobrachium* has a CPT with a MW similar to that in mammals and insect with epitopes that are recognized by mammalian anti-CPT I antibodies, indicating some sequence homology. The present work provides some indications about the mechanism through which fatty acids are channeled for catabolism in crustaceans. The low sensitivity towards malonyl-CoA-induced inhibition may indicate a sharing of control between CPT I and other enzymes, as has also been suggested in mammals (Eaton, 2002). In this regard it has been proposed that prawn ACS isoforms may be involved in the channeling of cytosolic fatty acids toward lipid synthesis or catalysis (Lavarías et al., 2007). The likely sharing of control between CPT I and other enzymes may allow for flexible regulation of metabolism and the ability to rapidly adapt β-oxidation flux to differing midgut gland requirements.

In this study, we have provided the first evidence that prawn present mitochondrial enzymes of a fatty acid shuttle system similar to that of vertebrates which may also be characteristic of other crustaceans, though more work is still needed on the role of this pathway in the regulation of overall mitochondrial fatty acid oxidation.

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