Topological and functional analysis of the rat liver carnitine palmitoyltransferase 1 expressed in *Saccharomyces cerevisiae*

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Abstract The rat liver carnitine palmitoyltransferase 1 (L-CPT 1) expressed in *Saccharomyces cerevisiae* was correctly inserted into the outer mitochondrial membrane and shared the same folded conformation as the native enzyme found in rat liver mitochondria. Comparison of the biochemical properties of the yeast-expressed L-CPT 1 with those of the native protein revealed the same detergent lability and similar sensitivity to malonyl-CoA inhibition and affinity for carnitine. Normal Michaelis-Menten kinetics towards palmitoyl-CoA were observed when careful experimental conditions were used for the CPT assay. Thus, the expression in *S. cerevisiae* is a valid model to study the structure-function relationships of L-CPT 1.

Key words: Carnitine palmitoyltransferase; Outer mitochondrial membrane; Topology; Functional study; Yeast expression

1. Introduction

The carnitine palmitoyltransferase (CPT; EC 2.3.1.21) enzyme system, in conjunction with carnitine/acylcarnitine translocase, provides the mechanism whereby long-chain fatty acids are transferred from the cytosol to the mitochondrial matrix to undergo β-oxidation (reviewed in [1]). The CPT system consists of two mitochondrial membrane-bound enzymes, CPT 1 and CPT 2. CPT 1 is an integral protein of the outer mitochondrial membrane (OMM), it is specifically inhibited by malonyl-CoA, the first committed intermediate of fatty acid biosynthesis, and it loses activity upon solubilization of mitochondria by strong detergents. By contrast, CPT 2 is located on the inner face of the inner mitochondrial membrane (IMM), is insensitive to malonyl-CoA inhibition and is released in active soluble form by detergents (reviewed in [2]). Inhibition of CPT 1 by malonyl-CoA plays a major role in the physiological regulation of fatty acid oxidation [1]. Only one CPT 2 isoform has been found, whereas two isoforms of CPT 1, encoded by distinct genes, have been described: the liver form (L-CPT 1) and the muscle form (M-CPT 1) (reviewed in [2]). Unlike M-CPT 1, L-CPT 1 displays altered sensitivity to malonyl-CoA under different physiopathological conditions (reviewed in [1]).

Elucidation of the molecular mechanisms involved in CPT 1 regulation by malonyl-CoA would be greatly facilitated by the development of an appropriate expression system that permits structure-function analysis of this key regulatory enzyme. Despite its usefulness for certain purposes [3,4], overexpression of L-CPT 1 in COS-1 cells is not suitable for functional studies, as these cells have endogenous CPT 1 and 2 activities. Although transfection in COS-1 cells has been used to analyze the biochemical properties of human CPT 2 mutants [5,6], these kinetic studies must be interpreted with caution due to the presence of endogenous CPTs. The yeast system is a more suitable model because of the complete absence of endogenous CPT [7]. Rat liver CPT 1 has been previously expressed in different strains of yeast [7,8]. However, in the first study the level of expression in *Saccharomyces cerevisiae* was very low [7] and in the second study the functional analysis (K_m for the substrates, IC_{50} for malonyl-CoA) of the enzyme expressed in *Pichia pastoris* was crude and never compared with the one of the native L-CPT 1 present in rat liver mitochondria [8]. For example, it was not known whether the rat L-CPT 1 expressed in yeast was correctly targeted to the OMM and adopted a conformation similar to the native enzyme. In addition, non-Michaelis-Menten kinetics towards palmitoyl-CoA of the rat L-CPT 1 expressed in yeast were reported [8]. The inability to measure the apparent K_m for palmitoyl-CoA of the yeast-expressed L-CPT 1 prevents further structure-function analysis such as determination of the amino acid residues involved in binding the palmitoyl-CoA substrate. The aim of the present study was to determine whether rat L-CPT 1 expression in *S. cerevisiae* could be improved and, secondly, to perform a more precise validation of this yeast expression model by determining if the yeast-expressed enzyme does indeed have the same topological and biochemical properties as the native protein.

2. Materials and methods

2.1. Plasmid construction for yeast expression of the rat L-CPT 1 and transformation of yeast cells

The cDNA for rat L-CPT 1 was excised from the pYes2.0-CPT1 vector [7] using restriction enzyme *Eco*RI and inserted into the pGEM4 plasmid (pGEM4-CPT1). This L-CPT 1 cDNA contains a 3′ non-coding region that was further deleted for yeast expression. pGEM4-CPT1Δ3′ was formed by deletion of the small *Sal*I fragment (encoding amino acids 669–773 and containing the stop codon and the 3′ non-coding region). The polymerase chain reaction (PCR) was then performed to copy a 338-nucleotide stretch of the 3′ coding sequence by using the 5′ primer (5′-TATGTGGTGTCCAAGTAT-3′) located upstream the unique *Sal*I restriction site of L-CPT 1 cDNA and the 3′ primer (5′-CCCGCTCGAGTTACTTTTTAGAATTGAT-3′) containing the stop codon and a *Xho*I restriction site. Replacement of the original *Sal*I fragment of the pGEM4-CPT1 with this PCR frag-

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Abbreviations: L-CPT 1, liver carnitine palmitoyltransferase 1; IMM, inner mitochondrial membrane; IPTG, isopropyl-β-D-thiogalactoside; OMM, outer mitochondrial membrane; STI, soybean trypsin inhibitor

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ment cut by *Sal*I and *Xho*I resulted in the generation of the pGEM4-CPT1Δ3′ plasmid.

For expression in *S. cerevisiae*, L-CPT 1 cDNA was excised from pGEM4-CPT1Δ3′ using the *Eco*RI and *Hin*dIII restriction enzymes. The *Eco*RI-blunted *Hin*dIII fragment was inserted into the yeast expression pYeDP1/8-10 vector [9] cut by *Eco*RI and *Sma*I. The L-CPT 1 cDNA was placed immediately downstream of the inducible *GAL10* promoter. The resulting plasmid was used to transform *S. cerevisiae* (haploid strain W303: *MAT*a, *his3*, *leu2*, *trp1*, *ura3*, *ade2-1*, *can1-100*) according to [10]. The multicopy pYeDP1/8-10 plasmid contains the *URA3* gene, allowing selection of transformants by plating on a minimal medium lacking uracil. The transformed colonies were then grown on liquid selecting medium in the presence of 2% galactose and harvested during the exponential growth phase. Crude cell extracts were prepared [11] and used for Western blotting experiments in order to confirm the presence of the expressed L-CPT 1. The control yeast cells used in this study were transformed with the pYeDP1/8-10 vector alone.

2.2. Yeast culture, isolation of yeast mitochondria and subcellular fractionation

Yeast strains were cultivated on selective minimal lactate medium (0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, 0.1% casamino acids, 2% lactate, 40 mg/ml adenine sulfate, 40 mg/ml L-tryptophan) at 30°C with shaking. To induce L-CPT 1 expression, 2% galactose was inoculated during the exponential growth and 10 h later the cells were harvested by centrifugation for 10 min at $3800\times g$.

Mitochondria of *S. cerevisiae* were prepared by differential centrifugations. Cells (6–10 g, wet weight) were washed once in 100 ml of water, resuspended in 50 ml of Tris-SO_4 buffer (0.1 M Tris-SO_4 pH 9.4; 10 mM DTT) and incubated for 10 min at 30°C with shaking. After a wash in 50 ml of sorbitol buffer (1.2 M sorbitol; 20 mM KPi; pH 7.4), cells were resuspended in sorbitol buffer (6 ml/er g of original pellet) and converted to spheroplasts by adding 3 mg of Zymolase 20T per g of cells and the mixture was incubated at 30°C with shaking for 30–45 min. All subsequent steps were performed at 4°C. The spheroplasts were sedimented by centrifugation at $3800\times g$ for 10 min, washed with sorbitol buffer and resuspended in 40 ml of SH buffer (0.6 M sorbitol; 20 mM HEPES-KOH; pH 7.4) containing 1 mM PMSF. After homogenization using 15 strokes of a Dounce homogenizer, samples were centrifuged at $2000\times g$ for 5 min. The supernatant was decanted and the pellet was similarly rehomogenized and centrifuged. The supernatants were combined and centrifuged at $17\,000\times g$ for 12 min. The crude mitochondrial pellet was washed once and finally resuspended in SH buffer. To determine the subcellular localization of the expressed L-CPT 1, the $17\,000\times g$ post-mitochondrial supernatant was further centrifuged at $150\,000\times g$ for 1 h at 4°C. Aliquots of the $150\,000\times g$ pellet and supernatant, and also of the starting homogenate and mitochondrial fraction, were then analyzed by SDS-PAGE and immunoblotting.

2.3. Submitochondrial localization of the yeast-expressed L-CPT 1

Hypotonic swelling of mitochondria was performed as previously described [12] with the following modifications. Mitochondria were resuspended in the homogenization buffer at a concentration of 50 μg protein/100 μl and diluted 10-fold in 20 mM HEPES (pH 7.4) in the absence or presence of 10 or 50 μg/ml trypsin. Control mitochondria (non-swelling conditions) were diluted to the same extent in SH buffer and also subjected to trypsin treatment, when indicated. Samples were kept on ice for 30 min and soybean trypsin inhibitor (STI) was added in a 20-fold excess. Mitoplasts/mitochondria were reisolated and washed once with SH buffer containing 1 mM EDTA and then lysed directly in Laemmli buffer. Samples were then analyzed by SDS-PAGE and immunoblotting.

2.4. Generation of anti-L-CPT 1 antibody

Antibody against amino acids 317–430 of the rat L-CPT 1 was raised as follows. The *Bgl*II-*Bam*HI fragment of L-CPT 1 cDNA encoding these amino acids was excised from pGEM4-CPT1 plasmid and subcloned into the pGEX-3X vector (Pharmacia) cut by *Bam*HI. The resulting plasmid encodes the GST protein fused at its C-terminus to amino acids 317–430 of L-CPT 1. This plasmid was used to transform the bacterial strain BL21 and the fusion proteins were induced for 2 h with isopropyl-β-D-thiogalactoside (IPTG; 0.1 mM) and purified by affinity chromatography on glutathione Sepharose beads according to the manufacturer's instructions. GST moieties were cleaved from the purified fusion proteins by factor Xa cleavage and the L-CPT 1 peptide was separated by SDS-PAGE electrophoresis and transferred onto nitrocellulose. The nitrocellulose band containing the L-CPT 1 peptide was used to immunize New Zealand white rabbits following standard procedures.

2.5. Western blot analysis

Aliquots of proteins were subjected to SDS-PAGE [13] in an 8% gel. The detection of proteins after blotting onto nitrocellulose was performed as described previously [12] using the ECL detection system (Pierce) according to the supplier's instructions. The antisera used were against the yeast cytochrome b_2 (1/1000), the yeast mitochondrial HSP70 (1/5000), the yeast porin (1/1000), the rat L-CPT 1 (1/3000) and the rat CPT 2 (1/1000).

2.6. Isolation of rat liver mitochondria and digitonin fractionation

Rat liver mitochondria were isolated from Wistar rats (200–300 g) bred in our laboratory and fed ad libitum on standard laboratory chow diet (62% carbohydrate, 12% fat and 26% protein in terms of energy). Mitochondria were isolated and further purified on self-forming Percoll gradients according to [14]. Purified mitochondria were resuspended in the isolation buffer (0.3 M sucrose; 5 mM Tris-HCl; 1 mM EGTA; pH 7.4).

Digitonin fractionation was performed as follows: 13 μl of mitochondria (at a concentration of 10 mg protein/ml) was added to 7 μl of buffer A (250 mM sucrose; 10 mM HEPES; 80 mM KCl; pH 7.6) containing varying concentrations of digitonin. Samples were incubated for 3 min at 4°C, diluted with 160 μl of buffer A supplemented with trypsin (10 μg/ml final concentration) and further incubated for 15 min at 30°C. Protease digestion was stopped with STI (20-fold excess). After a 10 min incubation at 4°C, the mitochondria were recovered by centrifugation, washed once with buffer B (250 mM sucrose; 10 mM HEPES; 1 mM EDTA; pH 7.6) and analyzed by SDS-PAGE and immunoblotting.

2.7. CPT assay

CPT activity was assayed at 30°C as palmitoyl-L-[*methyl*-^{3}H]carnitine formed from L-[*methyl*-^{3}H]carnitine (200 μM; 10 Ci/mol) and palmitoyl-CoA (80 μM) in the presence of 1% bovine serum albumin (w/v) as previously described [15]. Malonyl-CoA concentration varied over 0.01 to 150 μM for estimation of the IC_{50} value. The apparent K_m for carnitine was measured at 600 μM palmitoyl-CoA with 10–800 μM carnitine. For determination of the K_m for palmitoyl-CoA, carnitine concentration was 200 μM and palmitoyl-CoA concentration varied over 20 to 900 μM either in the presence of a fixed albumin amount (1% w/v) or in the presence of a fixed molar ratio of palmitoyl-CoA/albumin (6.1). When the CPT assay was performed using detergent-solubilized mitochondria, mitochondria were solubilized by 5% Triton X-100 as described in [8]. The insoluble membrane residue was sedimented by centrifugation at $16\,000\times g$ for 30 min at 4°C and the supernatant was used for the CPT assay. No CPT activity could be detected in the residual pellet fraction after solubilization.

2.8. Miscellaneous and chemicals

Protein concentration was determined by the Lowry procedure [16]. The fidelity of the PCR and the quality of DNA subcloning were confirmed by DNA sequence analysis. All restriction enzymes and T4 DNA ligase were purchased from Gibco (Life Technologies, France). The PCR reagents and T4 DNA polymerase were from New England Biolabs. Yeast culture media products were from Difco and Zymolase 20T from ICN Biomedicals France. L-[*methyl*-^{3}H]Carnitine was from Amersham and others chemicals were from Sigma.

3. Results and discussion

3.1. Rat liver CPT 1 expression in S. cerevisiae and subcellular localization

In an initial series of experiments, we determined whether the level of expression of the rat L-CPT 1 in the yeast *S. cerevisiae* could be increased by comparison to a previous

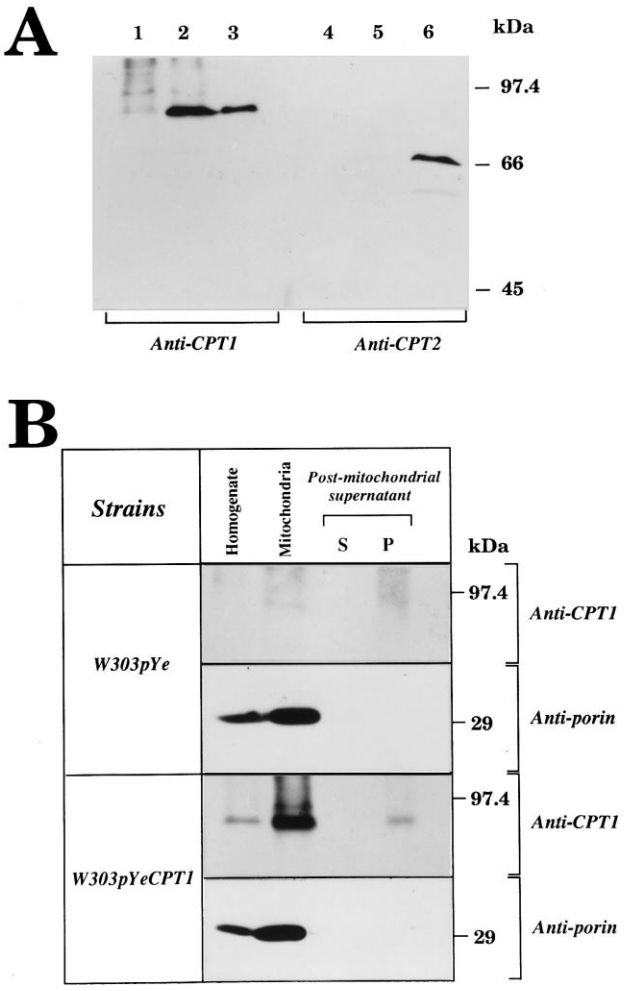

Fig. 1. Heterologous expression of the rat L-CPT 1 in *S. cerevisiae*. A: Immunodetection of the rat L-CPT 1 expressed in *S. cerevisiae* using an anti-L-CPT 1 antibody. A SDS-PAGE gel was run using paired amounts of protein from either homogenate of yeast cells (40 μg of protein) or isolated mitochondria from adult fed rat (80 μg of protein). Proteins were transferred onto nitrocellulose and one half of the blot was probed with an antibody against the rat L-CPT 1 (lanes 1–3) whereas the other half was probed with an antibody against the rat CPT 2 (lanes 4–6). Lanes 1 and 4: homogenate from control yeast cells; lanes 2 and 5: homogenate from yeast expressing the rat L-CPT 1; lanes 3 and 6: purified rat liver mitochondria. B: Subcellular localization of L-CPT 1 expressed in *S. cerevisiae*. Subcellular fractions were prepared from control yeast cells (W303pYe) and cells transformed with pYeCPT1 (W303pYeCPT1) as described in Section 2. 50 μg of protein from each subcellular fraction was analyzed by SDS-PAGE followed by immunoblotting with anti-L-CPT1 antibody. The quality of the subfractionation was assessed by immunoblotting with an anti-yeast porin antibody.

study [7]. We decided to delete the 3′ non-coding region present in the original L-CPT 1 cDNA. Indeed, efficient expression in yeast requires the deletion of the 5′ and 3′ non-coding regions originally present in the heterologous cDNA before its insertion into a yeast transcription unit [17]. The pYeDP1/8-10 expression vector was chosen because of its previously reported ability to express another mammalian OMM protein in *S. cerevisiae* [18]. In yeast cells grown in the presence of glucose, a large number of genes including several encoding mitochondrial respiratory enzymes are repressed [19,20], thus affecting mitochondrial biogenesis [21,22]. Therefore, yeast cells were grown on lactate as a non-fermentable carbon source before galactose induction of L-CPT 1.

Fig. 1A shows that our antibody raised against the rat L-CPT 1 strongly recognized the 88 kDa L-CPT 1 protein on Western blots of purified rat liver mitochondria and failed to detect any protein in the region of 68 kDa which is the size of the rat CPT 2 protein [23] (lane 3). This illustrates that the anti-L-CPT 1 antibody is specific and does not cross-react with the rat CPT 2 protein. This specific L-CPT 1 antibody could readily detect a 88 kDa protein in the homogenate of yeast cells transformed with pYeCPT1 and no specific signal was detected in the region of 88 kDa in control yeast cells transformed with the empty vector (Fig. 1A, compare lanes 1 and 2). Moreover, the immunoreactive protein expressed in yeast co-migrated with the native rat L-CPT 1 (Fig. 1A, compare lanes 2 and 3) suggesting that L-CPT 1 was indeed expressed in yeast. In support of this, antibody raised against the rat CPT 2 recognized the CPT 2 protein in purified rat liver mitochondria (Fig. 1A, lane 6) but failed to recognize the

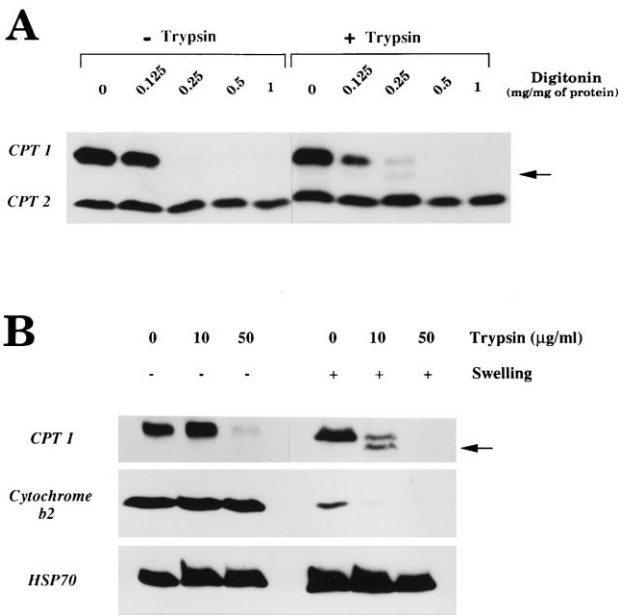

Fig. 2. Native and *S. cerevisiae*-expressed L-CPT 1 shared the same folded conformation. A: Trypsin proteolysis of intact or digitonin-treated rat liver mitochondria. Intact rat liver mitochondria were incubated for 3 min at 4°C in the presence of an increasing digitonin concentration (see Section 2). After dilution, samples were further incubated either in the absence or in the presence of trypsin (10 μg/ml) for 15 min at 30°C. Then soybean trypsin inhibitor was added and the samples were sedimented, washed and electrophoresed on SDS-PAGE. Western blot analysis was performed using anti-L-CPT 1 and anti-CPT 2 antibodies. Results are representative of three experiments. The arrow indicates a 83 kDa L-CPT 1 fragment generated by trypsin proteolysis in digitonin-treated mitochondria. B: Submitochondrial localization and topology of the L-CPT 1 expressed in *S. cerevisiae*. Mitochondria isolated from yeast expressing L-CPT 1 were incubated for 30 min at 4°C under either iso- (−swelling) or hypoosmotic (+swelling) conditions in the absence or in the presence of trypsin (10 or 50 μg/ml). After addition of soybean trypsin inhibitor, samples were sedimented, washed, electrophoresed on SDS-PAGE and analyzed by Western blot. Marker proteins for the different yeast mitochondrial subcompartments were cytochrome b_2 for the intermembrane space and mitochondrial HSP70 for the soluble matrix proteins. Results are representative of four different experiments. The arrow indicates a 83 kDa L-CPT 1 fragment generated by trypsin proteolysis in swollen yeast mitochondria.

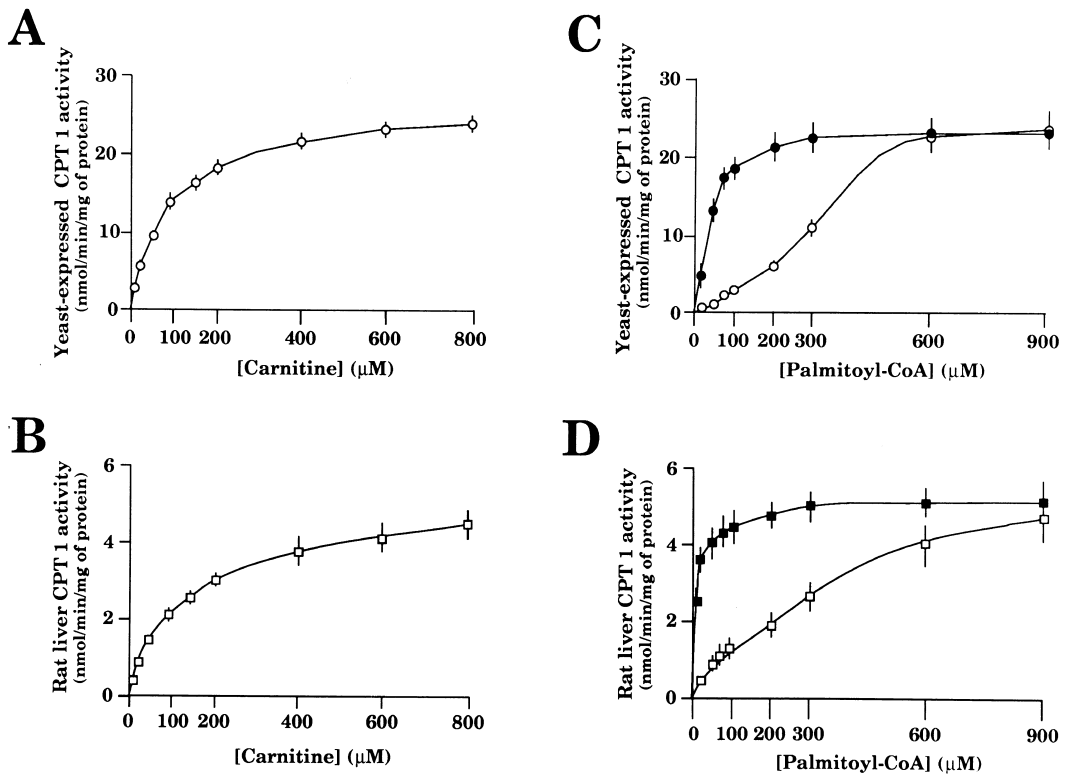

Fig. 3. Carnitine and palmitoyl-CoA saturation curves for the native and *S. cerevisiae*-expressed L-CPT 1. Intact mitochondria (50 μg) isolated from yeast expressing L-CPT 1 (○, ●) or from adult fed rat liver (□, ■) were assayed for CPT activity either at 600 μM palmitoyl-CoA with increasing concentrations of carnitine (A and B) or at 200 μM carnitine with increasing concentrations of palmitoyl-CoA (C and D). CPT assay was performed either in the presence of a fixed albumin concentration (1% w/v; ○, □) or a fixed molar ratio of palmitoyl-CoA/albumin (6.1:1; ●, ■). Results are means ± S.E.M. of four separate experiments.

yeast-expressed L-CPT 1 (Fig. 1A, lane 5). Subcellular fractionation showed that L-CPT 1 expressed in yeast was localized exclusively to the mitochondrial pellet fraction, as was true for another OMM protein, the yeast porin (Fig. 1B). Thus, the present study clearly shows that *S. cerevisiae* can be used to obtain a significant level of L-CPT 1 protein that is correctly targeted to yeast mitochondria. The very low level of expression of L-CPT 1 in *S. cerevisiae* reported earlier [7] was likely due to different experimental procedures for yeast expression and/or to the presence of the 3′ non-coding region in the L-CPT 1 cDNA used in these studies.

3.2. Submitochondrial localization of the yeast-expressed L-CPT 1

A recent topological analysis indicated that rat L-CPT 1 is an integral OMM protein with two transmembrane domains, both the N- and C-termini being exposed on the cytosolic side of the OMM [24]. The second aim of the present study was to determine if L-CPT 1 expressed in *S. cerevisiae* was inserted into the OMM in a conformation similar to that of the native enzyme despite possible changes in the phospholipid composition and/or fluidity of the OMM between these two species.

Experimental conditions were first designed to verify whether endogenous rat L-CPT 1 was resistant to a low trypsin concentration in intact mitochondria but not in digitonin-treated mitochondria. When intact rat liver mitochondria were incubated at 30°C for 15 min, L-CPT 1 was resistant to 10 μg/ml of trypsin (Fig. 2A) but was digested by higher trypsin concentrations (data not shown). Treatment of these mitochondria with 0.25 mg of digitonin/mg of protein resulted in solubilization of the OMM, as shown by the disappearance of L-CPT 1 protein (Fig. 2A). The IMM remained intact in the presence of 0.25–1.0 mg of digitonin/mg of protein since rat liver CPT 2 sedimented with the digitonin-permeabilized mitochondria (Fig. 2A). Rat liver CPT 2 was solubilized only at higher digitonin concentrations (above 2 mg of digitonin/mg of protein; data not shown). When the fractionation was performed in the presence of trypsin (10 μg/ml), L-CPT 1 was partially degraded into a 83 kDa fragment, but only when the OMM was disrupted (Fig. 2A). These results support a previous study [24] indicating that native L-CPT 1 adopts a highly folded state when anchored in the OMM and that sites sensitive to low concentrations of trypsin become accessible only when the OMM is disrupted.

To examine the submitochondrial localization of the L-CPT 1 expressed in yeast, intact mitochondria or hypotonic swollen mitochondria (mitoplasts) were subjected to trypsin treatment. Swelling experiments were performed rather than digitonin fractionation because experimental procedures for yeast mitochondria swelling were already established [12]. L-CPT 1 expressed in intact yeast mitochondria was resistant to a low trypsin concentration (10 μg/ml) but was nearly completely degraded by 50 μg/ml of trypsin without generation of any detectable proteolytic fragments (Fig. 2B). The yeast OMM was intact since endogenous cytochrome b_2, a soluble intermembrane space protein, was not degraded in the presence of trypsin (Fig. 2B). Hypotonic swelling of mitochondria was efficient since at least 90% of the cytochrome b_2 protein was

released and the remainder was digested by trypsin. By contrast, a mitochondrial matrix protein, HSP70, was still present in the mitoplasts and was not digested by trypsin (Fig. 2B). Under swelling conditions, and in the presence of 10 μg/ml of trypsin, L-CPT 1 expressed in yeast was cleaved to a 83 kDa fragment that was similar in size to that observed in the rat liver digitonin-disrupted mitochondria (Fig. 2A). These results indicate that L-CPT 1 expressed in yeast was correctly targeted and inserted into in the OMM, and adopted the same folded conformation as the native rat L-CPT 1, at least in terms of behavior towards trypsin treatment.

3.3. Functional analysis of the yeast-expressed L-CPT 1

The next step was to compare the biochemical properties of L-CPT1 expressed in *S. cerevisiae* with those of the native enzyme. Malonyl-CoA-sensitive CPT activity was observed only in mitochondria isolated from cells transformed with pYeCPT 1 (Table 1), confirming that *S. cerevisiae* mitochondria were devoid of endogenous CPT activity [7]. CPT activity in mitochondria from the transformed cells was 240% higher than in those isolated from the liver of fed rats (Table 1). Moreover, 93% of the CPT activity present in yeast mitochondria was inhibited by 150 μM malonyl-CoA (Table 1). As expected [7,8], detergent solubilization completely abolished the malonyl-CoA-sensitive enzyme expressed in yeast mitochondria and left essentially no residual CPT activity (Table 1). These observations clearly showed that the protein expressed in *S. cerevisiae* was indeed L-CPT 1. By contrast, CPT activity measured in detergent-solubilized rat liver mitochondria was increased in comparison to that monitored in intact organelles (Table 1). This results from the fact that, despite inactivation of L-CPT 1 by detergents, active soluble CPT 2 is released from detergent-solubilized rat liver mitochondria [23]. However, in this case a small residual sensitivity to malonyl-CoA was observed (Table 1), as reported previously in studies with rat liver mitochondria [25] and yeast cells expressing CPT 2 [8]. Its significance is still unclear. Importantly, in the present study, the IC_{50} value for malonyl-CoA of L-CPT 1 expressed in yeast was similar to the one found for the native enzyme (0.67 ± 0.13 μM for yeast versus 1.05 ± 0.23 μM for rat liver). This is the first study that reports clearly similar sensitivity to malonyl-CoA inhibition for both the yeast-expressed and native rat L-CPT 1 despite possible changes in the phospholipid composition and/or fluidity of the OMM between these two species. It has been proposed that variations in the sensitivity of L-CPT 1 to malonyl-CoA inhibition that occur in vivo may result from changes in conformation of the enzyme (reviewed in [1]). Thus, the observed similarity in the IC_{50} value of the native and yeast-expressed proteins strengthens the view that both products adopt a similar conformation.

3.4. Substrate saturation curves for the yeast-expressed L-CPT 1

Both yeast-expressed (Fig. 3A) and native (Fig. 3B) L-CPT 1 exhibited normal saturation kinetics with respect to carnitine. In yeast mitochondria expressing L-CPT 1, the calculated V_{max} and the apparent K_m for carnitine were respectively 26.4 ± 0.5 nmol/min/mg of protein and 87 ± 3 μM, whereas, when measured in rat liver mitochondria, the values were respectively 4.7 ± 0.5 nmol/min/mg of protein and 95 ± 2 μM. Thus, similar affinities for carnitine were observed for both native and yeast-expressed L-CPT 1 and the five-fold increase in the V_{max} for carnitine of the yeast-expressed L-CPT 1 illustrates the efficiency of the expression in yeast. It was reported recently that both rat liver CPT 1 and CPT 2 expressed in the yeast *P. pastoris* exhibited non-Michaelis-Menten saturation kinetics in response to an increasing palmitoyl-CoA concentration (20–300 μM) [8]. As seen from Fig. 3C, over a larger range of palmitoyl-CoA concentrations in the presence of 1% albumin, the kinetic curve was in fact sigmoidal, in keeping with previous observations [26–28]. Under these conditions, it is not possible to determine the K_m or V_{max} for palmitoyl-CoA. Although the allosteric behavior of L-CPT 1 towards its substrates and its inhibitor is still a subject of controversy, artefactual sigmoidicity has been attributed to substrate binding to albumin [28,29]. At low palmitoyl-CoA concentrations, binding sites on albumin may compete very effectively for palmitoyl-CoA, resulting in an underestimation of the actual CPT 1 activity for the total concentration of palmitoyl-CoA in the assay medium. The problem could be solved by using a 6.1:1 molar ratio of palmitoyl-CoA/albumin [28]. When we perform the CPT assay under these conditions, L-CPT 1 expressed in yeast exhibited a normal hyperbolic saturation kinetic curve for palmitoyl-CoA (Fig. 3C). From the Lineweaver-Burk plot, which was now linear, an apparent K_m (45 ± 7 μM) and V_{max} (25.7 ± 2.2 nmol/min/mg of protein) for palmitoyl-CoA could be determined. When the CPT assay was performed with rat liver mitochondria by using this 6.1:1 molar ratio of palmitoyl-CoA/albumin, the apparent K_m value for palmitoyl-CoA (16 ± 2 μM) and the V_{max} (5.2 ± 0.8 nmol/min/mg of protein) were very similar to those reported previously for the rat liver enzyme [30], but different from those obtained in yeast. Because the increased V_{max} of the L-CPT 1 expressed in yeast was accompanied by an increase in the apparent K_m for palmitoyl-CoA, mechanisms other than the presence of an increased amount of protein in yeast mitochondria have to be

Table 1
Characteristics of the L-CPT 1 activity expressed in yeast mitochondria

Stem	Intact mitochondria			Detergent-solubilized mitochondria		
	Malonyl-CoA		% inhibition	Malonyl-CoA		% inhibition
	−	+		−	+	
W303pYe	undetectable	undetectable	–	–	–	–
W303pYeCPT1	3.20 ± 0.20	0.20 ± 0.03	93.7 ± 0.8	0.023 ± 0.008	0.026 ± 0.004	0
Rat liver	1.31 ± 0.19	0.15 ± 0.02	88.6 ± 4.4	2.30 ± 0.20	1.68 ± 0.18	26.9 ± 6.2

Mitochondria were isolated from control yeast cells (W303pYe), yeast cells expressing L-CPT 1 (W303pYeCPT 1) and from fed rat liver. CPT activity was assayed with 80 μM palmitoyl-CoA and 200 μM carnitine in the absence or in the presence of 150 μM malonyl-CoA, using either intact mitochondria or mitochondria solubilized in 5% Triton X-100, as described in Section 2. The % inhibition of CPT activity by 150 μM malonyl-CoA is indicated. CPT activity is expressed in nmol/min/mg protein. Results are means ± S.E.M. of three to seven separate experiments.

considered. Since alterations in protein conformation seem unlikely (see above), a possible explanation could be the absence in yeast cells of a liver-specific modification involving membrane lipid environment and/or protein/protein interaction. It should also point out that when the CPT assay was performed with rat liver mitochondria by using 1% albumin and varied palmitoyl-CoA concentrations, the kinetic curve was not sigmoidal (Fig. 3D) by contrast to what observed for the L-CPT 1 expressed in yeast (Fig. 3C). This discrepancy between yeast-expressed and native L-CPT 1 may result from the decreased affinity for palmitoyl-CoA observed for the yeast-expressed L-CPT 1.

In summary, we demonstrate here that: (1) a high level of expression of an active rat L-CPT 1 can be obtained in *S. cerevisiae*, (2) the expressed protein is correctly targeted to the OMM and exhibits the same sensitivity to malonyl-CoA and membrane topology as the native enzyme and (3) kinetic parameters (K_m, V_{max}) of the yeast-expressed L-CPT 1 can be measured for both carnitine and palmitoyl-CoA with careful attention to the experimental conditions of the CPT assay, allowing further site-directed mutagenesis analysis of the amino acid residues involved in the interaction between L-CPT 1 and palmitoyl-CoA or carnitine. Thus, *S. cerevisiae* represents a valid model for the study of the structure-function relationships of L-CPT 1.

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