Functional Characterization of Mitochondrial Carnitine Palmitoyltransferases I and II Expressed in the Yeast *Pichia pastoris*[†,‡]

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ABSTRACT: The rate-limiting step in β oxidation is the conversion of long-chain acyl-CoA to acylcarnitine, a reaction catalyzed by the outer mitochondrial membrane enzyme carnitine palmitoyltransferase I (CPTI) and inhibited by malonyl-CoA. The acylcarnitine is then translocated across the inner mitochondrial membrane by the carnitine/acylcarnitine translocase and converted back to acyl-CoA by CPTII. Although CPTII has been examined in detail, studies on CPTI have been hampered by an inability to purify CPTI in an active form from CPTII. In particular, it has not been conclusively demonstrated that CPTI is even catalytically active, or whether sensitivity of CPTI to malonyl-CoA is an intrinsic property of the enzyme or is contained in a separate regulatory subunit that interacts with CPTI. To address these questions, the genes for CPTI and CPTII were separately expressed in *Pichia pastoris*, a yeast with no endogenous CPT activity. High levels of CPT activity were present in purified mitochondrial preparations from both CPTI- and CPTII-expressing strains. Furthermore, CPTI activity was highly sensitive to inhibition by malonyl-CoA while CPTII was not. Thus, CPT catalytic activity and malonyl-CoA sensitivity are contained within a single CPTI polypeptide in mammalian mitochondrial membranes. We describe the kinetic characteristics for the yeast-expressed CPTs, the first such report for a CPTI enzyme in the absence of CPTII. Yeast-expressed CPTI is inactivated by detergent solubilization. However, removal of the detergent in the presence of phospholipids resulted in the recovery of malonyl-CoA-sensitive CPTI activity, suggesting that CPTI requires a membranous environment. CPTI is thus reversibly inactivated by detergents.

Transport of long-chain fatty acids from the cytoplasm to the mitochondrion involves the conversion of their acyl-CoA derivatives to acylcarnitine, translocation across the inner mitochondrial membrane, and reconversion to acyl-CoA (Bieber, 1988; McGarry et al., 1989). These transferase reactions are the rate-limiting steps in β oxidation and require activity for carnitine palmitoyltransferase (CPT).[1] CPT activity is associated with two polypeptides, named CPTI and CPTII, located on the outer and inner mitochondrial membranes, respectively (Murthy & Pande, 1987; Bieber, 1988; McGarry et al., 1989; Ghadiminejad & Saggerson, 1991). A thorough understanding of the CPT system is an important first step toward developing treatments for diseases such as diabetes and myocardial ischemia (Corr & Yamada, 1995; Prentki & Corkey, 1996). For example, certain drugs used to treat diabetes specifically inhibit CPTI (Cook, 1987), and transition-state analogs of CPTI inhibitors act as antidiabetic agents (Anderson et al., 1995). In pancreatic β cells, inhibition of CPTI stimulates insulin secretion (Chen et al., 1994).

In spite of its importance, basic questions remain about the CPT system. Early studies showed that in intact rat liver mitochondria, CPT activity is greatly inhibited by malonyl-CoA and related compounds (Kiorpes et al., 1984; Declercq et al., 1985, 1987; Woeltje et al., 1987; Esser et al., 1993b). However, upon detergent solubilization of the mitochondrial membranes, CPT activity is still present but is no longer sensitive to malonyl-CoA (Bremer, 1981; Bremer et al., 1985; McGarry et al., 1989). Further investigations indicated that with intact organelles, CPT assays primarily detect a malonyl-CoA sensitive CPTI and that CPTII activity is latent (i.e., shielded from one or both substrates by the inner mitochondrial membrane). Upon membrane solubilization, CPTI is completely inactivated, but CPTII is not and is instead released from latency and becomes fully active (Bieber, 1988; McGarry et al., 1989). Because CPTII is detergent stable, it can be readily purified and has been studied in detail (Bieber, 1988; McGarry et al., 1989). In contrast, it has not been possible to purify active CPTI, and investigations of this enzyme have been largely limited to systems that also contain CPTII (Lund, 1987; Kerner & Bieber, 1990; Murthy & Pande, 1990; Kerner et al., 1994). As a result, CPTI is virtually defined as that portion of CPT activity that exists in intact mitochondria and is malonyl-CoA sensitive.

At least two major questions with regard to CPTI remain unresolved. Is CPTI a catalytically active distinct protein independent of CPTII, as described above? If so, is sensitivity to malonyl-CoA an intrinsic function of CPTI or

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[1] Abbreviations: *AOXp*, alcohol oxidase I promoter; CPT, carnitine palmitoyltransferase; *GAPp*, glyceraldehyde-3-phosphate dehydrogenase promoter; MBP, maltose binding protein; PCR, polymerase chain reaction; RLM, rat liver mitochondria.

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is it conferred by a separate distinct regulatory protein (McGarry et al., 1989; Chung et al., 1992; Saggerson et al., 1992; Woldegiorgis et al., 1992)? With regard to the latter question, detergent-solubilized rat liver mitochondria (RLM) contain a protein that binds malonyl-CoA and related compounds such as tetradecylglycidyl-CoA and etomoxiryl-CoA, and is distinct from the major CPT activity fraction (Woldegiorgis et al., 1985; Lund & Woldegiorgis, 1987; Chung et al., 1992; Saggerson et al., 1992). This binding protein has been identified as 90−94-kDa protein in liver and an 82-kDa protein in heart and skeletal muscle, but always without CPT activity (Kiorpes et al., 1984; Declercq et al., 1987; Esser et al., 1993b). In previous studies, we were able to restore malonyl-CoA sensitivity to solubilized and purified CPT by reconstitution with purified preparations of malonyl-CoA-binding protein using mitochondria from rat liver or heart, and we interpreted these results as evidence for a separate regulatory subunit (Chung et al., 1992; Woldegiorgis et al., 1992).

Recently, both the rat and human liver mitochondrial inactive CPTI cDNAs were cloned (Esser et al., 1993a; Britton et al., 1995). The cDNAs predicted that the coding sequence of inactive CPTI shared limited similarity with that of CPTII (Woeltje et al., 1990b). Transfection of COS cells with the rat liver cDNA resulted in induction of malonyl-CoA-sensitive CPT (Esser et al., 1993a). However, COS cells have endogenous malonyl-CoA-sensitive CPT which may have been induced. More recently, the same inactive rat liver CPTI cDNA was expressed in *Saccharomyces cerevisiae*, an organism devoid of endogenous CPT activity (Brown et al., 1994b). Although small amounts of inactive CPTI protein were detected in mitochondrial fractions, CPTI activity levels were barely detectable in crude extracts and were undetectable in mitochondrial fractions; thus, the existence of malonyl-CoA-sensitive CPTI could not be unequivocally demonstrated (Brown et al., 1994b). To resolve the question as to whether or not catalytic activity and malonyl-CoA sensitivity reside in a single CPT polypeptide, we expressed both CPTI and CPTII cDNAs from rat liver in the methylotrophic yeast *Pichia pastoris* and examined the activity, localization, and biochemical properties of the proteins.

EXPERIMENTAL PROCEDURES

Isolation of Rat Liver CPTI and CPTII cDNA Clones. cDNA clones encoding CPTI and CPTII were isolated from a rat liver cDNA library in λgt11 (Clontec, Palo Alto, CA) by plaque hybridization using PCR-amplified cDNA fragments as probes (Sambrook et al., 1989; Ochman et al., 1990). Pairs of PCR primers were designed based on published cDNA sequences (Woeltje et al., 1990b; Esser et al., 1993a) and were used to amplify short cDNA fragments (366 bp, CPTI; 285 bp, CPTII) from the cDNA library (Ochman et al., 1990). PCR primers for CPTI were the following: forward, 5′-ATGGCAGAGGCTCACCAAGCTGTGGCC-3′; reverse, 5′-CATGGTCATGATGACTGCCACCCAGAG. PCR primers for CPTII were as follows: forward, 5′-GATAAGCAGAATAAGCACACC-3′; reverse, 5′-GGAGGAACAAAGCGAATGAGT-3′.

Radioactive ([α-^{32}P]dCTP) probes were prepared from the fragments by the random-labeling method (Feinberg & Vogelstein, 1984). To isolate CPTI, ∼8×10^5 plaques were transferred to nitrocellulose and screened with the 366-bp probe to obtain 6 positive clones. For CPTII, 10 portions of the cDNA library, each containing ∼8×10^4 plaque-forming units, were amplified by allowing the bacteriophage to reproduce 100-fold in liquid culture. Samples of each pool were used as templates for PCR amplification using the CPTII-specific primers described above (Takumi & Lodish, 1994). Of the 10 pools, 1 was able to productively serve as the template, and 2.4×10^5 plaques from this subpool were transferred to nitrocellulose and screened with the CPTII probe to obtain 4 positive clones. Two of the six potential CPTI-containing clones and three of the four CPTII-containing clones contained at least a portion of the respective cDNAs. The identity of the cDNA inserts was further confirmed in one clone of each CPT gene by sequencing the inserts.

Generation of Anti-CPTI and Anti-CPTII Antibodies. Rabbit polyclonal antibodies were raised against bacterially expressed CPTI and CPTII using the maltose-binding protein (MBP) system (New England Biolabs, Beverly, MA). *Eco*RI fragments containing the CPT genes were prepared from the bacteriophage clones and subcloned into the pMAL-c2 vector to generate pM1R (CPTI) and pM2R (CPTII). A MBP−CPTI fusion construct encoding the C-terminus of CPTI (amino acid residues 651−773) was made by restriction digestion of pM1R with *Nae*I and *Eco*RI, and ligation of the 0.4-kb CPTI C-terminal encoding fragment into pMAL-c2 cut with *Xmn*I and *Eco*RI. A similar MBP−CPTII fusion encoding amino acid residues 541−658 of CPTII was constructed by restriction digestion of pM2R with *Pvu*II and *Pst*I followed by ligation of the 0.4-kb CPTII C-terminal encoding fragment into pMAL-c2 cut with *Xmn*I and *Pst*I. The plasmids were transformed into *Escherichia coli*, and the fusion proteins were induced with IPTG and purified by affinity chromatography on an amylose column according to the manufacturer's instructions. The purified fusion proteins were used to immunize rabbits following standard procedures (Josman Laboratories, Napa, CA).

Construction of Plasmids for CPT Expression in P. pastoris. An *Eco*RI site was introduced by PCR immediately 5′ of the ATG start codons of the CPTI and CPTII cDNAs to enable cloning into the unique *Eco*RI sites located just 3′ of the alcohol oxidase I gene promoter (*AOXp*) in plasmid pHIL-D2 (Invitrogen, San Diego, CA) and the glyceraldehyde-3−phosphate dehydrogenase gene promoter (*GAPp*) in plasmid pHWO10 (Waterham et al., 1996b).

For CPTI, PCR primers were designed to generate a 240-bp fragment with a *Hin*dIII and an *Eco*RI restriction site immediately 5′ of the ATG start codon in the CPTI cDNA. The reverse primer hybridized to a site 3′ of a unique *Sph*I site in CPTI. The PCR product was ligated as a *Hin*dIII−*Sph*I fragment into pUC119 containing the rest of the CPTI gene as a 2376-bp *Sph*I−*Eco*RI fragment. The resulting vector was digested with *Eco*RI to release a full-length modified CPTI fragment of 2606 bp which was then ligated into *Eco*RI-cut pHIL-D2 and pHWO10 to produce pYGW10 and pYGW11, respectively.

For CPTII, PCR primers were designed to amplify a fragment of 92 bp that contained an *Eco*RI restriction site immediately 5′ of the ATG start codon of CPTII. The reverse primer hybridized to a site 3′ of a unique *Afl*II restriction site within the gene and added a *Bam*HI to the 3′ terminus of the PCR product. The PCR fragment was digested with *Eco*RI and *Bam*HI and ligated into the same

sites in pUC119. The resulting plasmid was then cut with *Afl*II and *Hin*dIII. The remainder of the CPTII gene was released from pM2R as a 2500-bp *Afl*II−*Hin*dIII fragment and ligated into the pUC119 derivative containing the *Eco*RI−*Bam*HI PCR fragment. A 2245-bp *Eco*RI fragment from the plasmid containing the modified full-length CPTII gene was then ligated to an *Eco*RI-cut pHIL-D2 and pHWO10 to produce pYGW8 and pYGW6, respectively.

P. pastoris Transformations. The expression plasmids were linearized in their *HIS4* genes by digestion with *Bsp*EI (pYGW10 and pYGW9) or *Sal*I (pYGW8 and pYGW6) and integrated into the *HIS4* locus of *P. pastoris* strain GS115 (*his4*) by electrotransformation (Becker & Guarente, 1991). Histidine prototrophic transformants were selected on YND plates and grown in liquid YND medium (*GAPp*) or YNM medium (*AOXp*) [0.17% yeast nitrogen base without amino acids and ammonium sulfate (Difco Laboratories Inc., Detroit, MI) supplemented with 0.5% ammonium sulfate and 0.5% (w/v) glucose or 0.5% (v/v) methanol, respectively] and harvested during the exponential growth phase (Liu et al., 1992). Crude cell extracts were prepared by disrupting the cells with glass beads and then assaying for CPT activity as previously described (Bremer et al., 1985).

Isolation of Yeast Mitochondria. P. pastoris cells were spheroplasted, lysed, and homogenized, and the resulting extracts were subjected to differential centrifugation by a modification of the procedure described by Liu et al. (1992). Cultures of ∼800−1600 OD_{600} units were harvested by centrifugation at 5000*g* for 10 min at 4 °C, and cells were washed 3 times with cold deionized H_2O followed by suspension in 10 mL of spheroplast buffer [5 mM MOPS, 0.5 M KCl, and 10 mM Na_2SO_3 (pH 7.2)] per 500 OD_{600} units. Cells were converted to spheroplasts by adding 0.8 mg of Zymolase 100T (Seikagaku America Inc., Ijamsville, MD) per 500 OD_{600} units and incubated at 30 °C with gentle shaking for 15−45 min. All subsequent steps were performed at 4 °C. The spheroplasts were sedimented by centrifugation at 3000*g* for 8 min and washed with 10 mL of spheroplast buffer per 500 OD_{600} units containing 1 mM PMSF. The washed spheroplasts were then suspended in 5 mL of homogenization buffer [5 mM MES, 0.5 mM EDTA, 1.2 M sorbitol, 0.1% ethanol, 0.21 mg/mL NaF (pH 6.0), 1 mM PMSF, and 1.25 μg/mL leupeptin] per 500 OD_{600} units and homogenized for 10 cycles in a glass Potter Elvehjem tissue grinder held on ice with a tight-fitting pestle. The homogenate was centrifuged at 3000*g* for 10 min to remove whole cells, cell debris, and nuclei, and the supernatant was centrifuged at 30000*g* for 30 min. The resulting mitochondrial pellet was rinsed with 1 mL of homogenization buffer and resuspended in 0.5 mL of homogenization buffer per 500 OD_{600} units. Mitochondrial preparations were immediately assayed for malonyl-CoA-sensitive CPTI activity. For CPTII assays, mitochondrial suspensions in homogenization buffer were solubilized by addition of octyl glucoside to 1% final concentration, and the mixtures were incubated for 30 min on ice with occasional mixing. Unless otherwise indicated, the solubilized mitochondrial preparations were immediately assayed for CPTII activity. To determine the subcellular localization of CPTI and CPTII, the organelle pellets were loaded on top of a sucrose density gradient (Waterham et al., 1996a) composed of 4 mL of 60%, 5 mL of 50%, 8 mL of 45%, 5 mL of 40%, 5 mL of 35%, and 4 mL of 32% (w/w) sucrose in homogenization buffer without PMSF, NaF, and leupeptin, and centrifuged at 4 °C for 6 h at 27 000 rpm in a Beckman SW27Ti rotor. Fractions of ∼1.2 mL were collected from the bottom of the tube and assayed for CPT (Bremer et al., 1985) and cytochrome *c* oxidase activities (Douma et al., 1985).

CPT Assay. CPT activity was assayed by the forward exchange method using L-[^{3}H]carnitine as previously described (Bremer et al., 1985). In a total volume of 0.5 mL, the standard enzyme assay mixture contained 0.2 mM L-[^{3}H]carnitine (∼10 000 dpm/nmol), 50 μM palmitoyl-CoA, 20 mM HEPES (pH 7.0), 1 or 2% fatty acid-free albumin, and 40−75 mM KCl, with or without 10−100 μM malonyl-CoA. Reactions were initiated by addition of mitochondria, membranes containing expressed proteins, detergent extracts, or proteoliposomes containing the reconstituted CPTI. The reaction was linear up to 4 min, and all incubations were done at 30 °C for 3 min. Reactions were stopped by addition of 6% perchloric acid and were then centrifuged at 2000 rpm for 7 min. The resulting pellet was suspended in water, and the product [^{3}H]palmitoylcarnitine was extracted with butanol at low pH. After centrifugation at 2000 rpm for 2 min, an aliquot of the butanol phase was transferred to a vial for radioactive counting.

Mitochondrial Membrane Solubilization. Yeast mitochondria containing the expressed CPTI or CPTII were sedimented by centrifugation at 16000*g* for 30 min at 4 °C, and the pellet was solubilized in 5% Triton X-100 (10 mg of protein/mL) containing 500 mM KCl and 10 mM HEPES (pH 7.0) at 0 °C for 30 min. The insoluble membrane residue was sedimented by centrifugation at 16000*g* for 30 min, and the supernatant was used for CPT assay, malonyl-CoA binding, and reactivation of malonyl-CoA-sensitive CPTI by reconstitution (detergent removal in the presence of phospholipids).

Reconstitution of Malonyl-CoA-Sensitive CPT Activity. A modified reconstitution procedure described by Garlid et al. (1995) was used. Membranes containing CPTI and CPTII were solubilized in Triton X-100 as described above. The dried phospholipids (50 mg) composed of asolectin and cardiolipin at a ratio of 9:1 were first suspended in 700 μL of HEPES (pH 7.0) containing 100 μL of octylPOE, followed by addition of 200 μL of the Triton X-100 extracts. The detergent−protein−lipid mixture was applied to a Biobeads SM-2 column (3 mL) and incubated for 90 min at 4 °C to remove the detergent. The proteoliposomes were recovered by low-speed centrifugation. Malonyl-CoA-sensitive CPT activity in the proteoliposomes was measured as described above.

Western Blot Analysis. Proteins were separated by SDS−PAGE (Laemmli, 1970) in a 7.5% gel and transferred onto nitrocellulose membranes using a Mini Trans-Blot Electrophoretic Transfer Cell according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Immunoblots were developed by incubations with the CPTI- and CPTII-specific polyclonal antibodies (1:10 000), followed by an anti-rabbit IgG conjugated to alkaline phosphatase. The antigen−antibody complex was detected using a Tropix Western Light chemiluminescence kit (Bedford, MA).

Miscellaneous Materials and Methods. DNA sequencing was done at the Oregon Regional Primate Research Center Core Facility using an automatic DNA sequencer (Sanger et al., 1977). Protein was determined by the Lowry procedure (Lowry et al., 1951).

All restriction enzymes were from New England Biolabs (Beverly, MA). The *E. coli* strain used was DH5$_\alpha$, and pUC119 was a gift from Dr. Matthew S. Sachs of the Oregon Graduate Institute of Science and Technology. L-[^{3}H]Carnitine was from Amersham (Arlington Heights, IL); nucleotides and palmitoyl-CoA were from Pharmacia (Piscataway, NJ); asolectin (45%) was from Avanti Polar Lipids, Inc. (Alabaster, AL); cardiolipin and octyl glucoside were from Sigma (St. Louis, MO); and Biobeads SM-2 was from BioRad (Hercules, CA).

RESULTS

Isolation of Rat Liver CPTI and CPTII cDNA Clones. Full-length RLM CPTI (2.7 kb) and CPTII (2.3 kb) cDNA clones were isolated by screening a rat liver cDNA library in λgt11 using CPTI- and CPTII-specific PCR-amplified DNA fragments. Since the library used for this study was different from that previously reported in the literature for isolation of CPTI and CPTII clones (Woeltje et al., 1990b; Esser et al., 1993a), we sequenced our cDNA clones and compared them with the published sequences. Our CPTI sequence predicted only one difference in amino acid sequence (I480V). For CPTII, seven differences in amino acid sequence between the proteins were evident (M135V, Q167R, V245I, L267S, D346Q, S373A, and T394S). Three of the predicted differences were conservative.

Expression of CPTI and CPTII in the Yeast P. pastoris. *P. pastoris* was chosen as an expression system for both CPTI and CPTII, because it does not have endogenous CPT activity. The *P. pastoris* expression plasmids were constructed to express CPTI and CPTII under control of *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase or alcohol oxidase I gene promoters, *GAPp* and *AOX1p*, respectively. *P. pastoris* strains transformed with the CPT expression vectors were grown in liquid media supplemented with either glucose (*GAPp*) or methanol (*AOXp*). As a control, yeast strains containing each vector, but without a CPT cDNA insert, were grown in parallel cultures. Crude cell extracts were prepared by disrupting the cells with glass beads and assaying for CPT activity. Most strains transformed with either a CPTI- or a CPTII-containing vector showed substantial amounts of CPT activity, while no activity was found in the non-CPT-containing control cells (data not shown). For further studies, we chose to work with strains that expressed CPTI and CPTII under control of *GAPp* for the following reasons: (1) CPT activities measured in glucose-grown transformants expressing either CPT cDNA under control of *GAPp* were similar to those of the methanol-grown transformants expressing either CPT cDNA under control of *AOXp*; (2) unlike methanol, glucose did not induce peroxisomes (organelles that copurify with mitochondria during differential centrifugation); (3) constitutive expression of CPTI and CPTII via the *GAPp* did not appear to be toxic to the cells; and (4) it was not necessary to shift cultures from glucose to methanol to induce expression.

Subcellular Localization of the CPTs. To determine the subcellular localization of the CPT enzymes in *P. pastoris*, homogenized spheroplasts derived from the expression strains were fractionated into mitochondrial (pellet) and cytoplasmic (supernatant) fractions, which were then assayed for cytochrome *c* oxidase (an inner mitochondrial membrane marker enzyme) and CPT. Activity for both CPTI and CPTII was found mainly in the pellet fractions as shown in Table 1. CPTII, an inner mitochondrial membrane enzyme in mammalian cells, was localized almost exclusively in the mitochondrial pellet fraction as was cytochrome *c* oxidase. With CPTI, an outer mitochondrial membrane protein in mammalian cells, about 80% of its activity was present in the mitochondrial pellet fraction, and the remaining 20% was in the cytoplasmic fraction. Sucrose density gradient profiles of the mitochondria-enriched pellets from CPTI (Figure 1A) and CPTII expression strains (Figure 1B) showed a distribution pattern for CPT activities that was nearly identical to that for cytochrome *c* oxidase, indicating that CPTI and CPTII were localized to the yeast mitochondria.

Table 1: Distribution of CPT and Cytochrome *c* Oxidase Activities in Yeast Strains Expressing CPTI and CPTII[a]

strain	enzyme	P[b]	S[b]	P/S[b]
WWG1-7	cytochrome *c* oxidase	1.19	0.01	119
	CTPI	6.83	1.38	4.95
WWG2-1	cytochrome *c* oxidase	0.99	0.03	33
	CPTII	3.18	0.01	318

[a] Activities were expressed in nmol of palmitoylcarnitine min^{-1} (mg of protein)$^{-1}$ for CPT and μmol of product min^{-1} (mg of protein)$^{-1}$ for cytochrome *c* oxidase. [b] Mitochondria-enriched pellet (P) and cytoplasmic supernatant fractions (S) were prepared by subcellular fractionation of glucose-grown yeast strains expressing CPTI or CPTII cDNA and assayed for cytochrome *c* oxidase and CPT activities, as described under Experimental Procedures.

Immunoblots with the CPTI- and CPTII-specific polyclonal antibodies showed the presence of 88-kDa and 70-kDa protein species corresponding to CPTI (Figure 2A) and CPTII (Figure 2B), respectively, in the mitochondria-enriched pellets and mitochondrial peak fractions of the sucrose density gradients of the strains that expressed CPTI and CPTII. CPT proteins of the same sizes were also seen in RLM, but not in the non-CPT cDNA-containing control strain. The cytoplasmic supernatant fraction from the yeast strain expressing CPTI also had a faint reactive protein at 88 kDa (Figure 2A), in agreement with the ~20% CPTI activity observed in this fraction in all our preparations. RLM and the yeast mitochondrial preparations had major cross-reacting low-molecular-weight proteins of unknown origin. This was most likely due to the presence of proteolytic fragments of CPTI and CPTII in RLM and the yeast preparations.

Biochemical and Kinetic Properties of Yeast-Expressed CPTI. Isolated mitochondria from the CPTI expression strain exhibited a high level of malonyl-CoA-sensitive CPT activity. Malonyl-CoA-sensitive CPT activity was only observed in cells transformed with CPTI cDNA but not in control cells lacking the cDNAs as shown in Table 2. CPTI activity was 92−96% inhibited by 100 μM malonyl-CoA in intact mitochondria with an I_{50} of 1.9 μM (Figure 3). Solubilization of the mitochondria in 5% Triton X-100 completely abolished CPT activity (Table 2), a further demonstration that the expressed protein is indeed CPTI. These observations clearly demonstrate that the catalytic activity and malonyl-CoA sensitivity reside in one polypeptide, and, furthermore, CPTI is detergent-labile.

Membrane-bound CPTI of yeast mitochondria exhibited normal saturation kinetics when the carnitine concentration was varied relative to a constant second substrate, palmitoyl-CoA, as shown in Figure 4A. The calculated K_m for carnitine

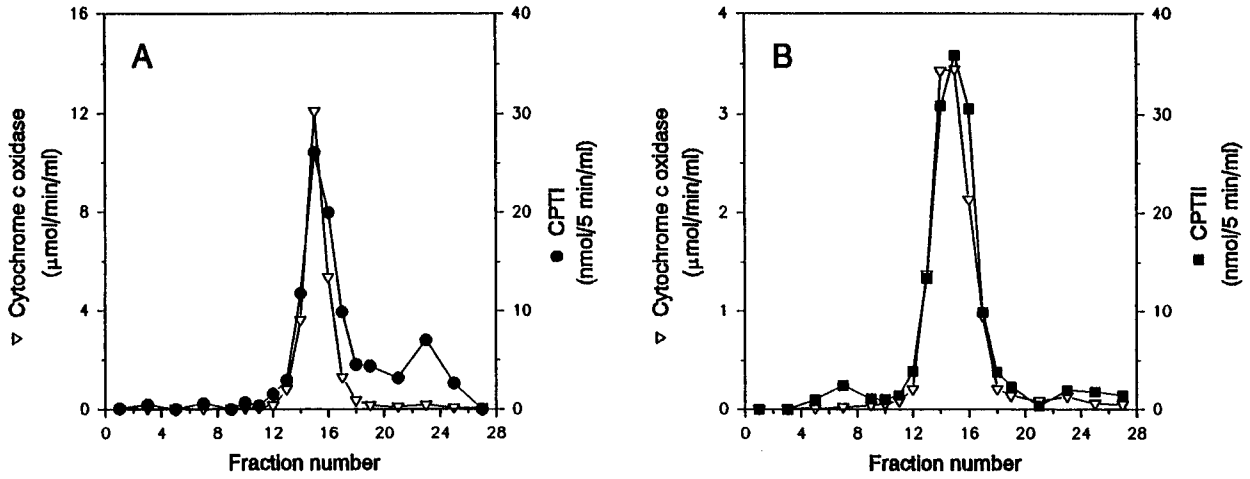

FIGURE 1: Distribution of CPT and cytochrome *c* oxidase activities after centrifugation of crude yeast mitochondrial preparations through sucrose gradients. Mitochondria-enriched pellets were prepared from glucose-grown yeast strains expressing CPTI (A) or CPTII (B) and subjected to sucrose density gradient centrifugation as described under Experimental Procedures. Fractions were then assayed for CPT and cytochrome *c* oxidase activities.

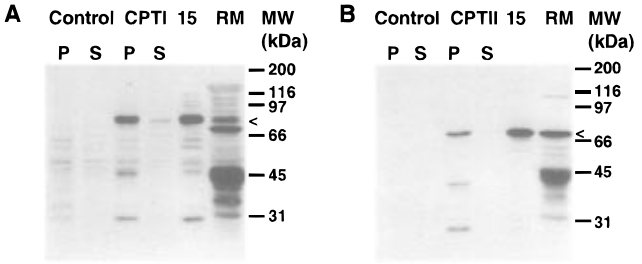

FIGURE 2: Immunoblots showing the distribution of CPTI (A) and CPTII (B) proteins in mitochondria-enriched pellet fractions (P), cytoplasmic supernatant fractions (S), and mitochondrial peak fractions (15) of the sucrose density gradients shown in Figure 1. Approximately 25 μg of protein was applied in each lane, except for the rat liver mitochondrial lane which had 130 μg of protein. The arrows identify the protein species recognized: 88 kDa for CPTI and 70 kDa for CPTII. Control = yeast strains with vectors but without a CPTI or CPTII insert.

was 32 μM. The V_{max} was 6.9 nmol min^{-1} (mg of protein)$^{-1}$. However, yeast-expressed CPTI demonstrated an abnormal response to increasing concentrations of palmitoyl-CoA. The reaction was linear up to 300 μM palmitoyl-CoA (Figure 4B). This abnormal behavior was also exhibited by yeast-expressed CPTII, as described below. These unusual responses are also observed to a lesser degree in RLM and may be due to the presence of albumin in the reaction mixture, the detergent properties of palmitoyl CoA, and/or to differences in the membrane environment of the enzymes in yeast mitochondria compared to RLM. Therefore, it was not possible to determine a K_m or V_{max} for palmitoyl-CoA.

Reconstitution of Active CPTI in Liposomes. Detergent solubilization of mitochondria from the CPTI-expressing strain abolished CPTI activity (Table 2). To reactivate the detergent-inactivated enzyme, a 5% Triton X-100 extract of CPTI-containing mitochondria was added to a detergent−phospholipid mixture, and the detergent−proteolipid mixture was passed through a Biobeads SM-2 column to remove the detergent and generate proteoliposomes. The resulting proteoliposomes exhibited malonyl-CoA-sensitive CPTI activity of up to 1.0 nmol (mg of protein)$^{-1}$ min^{-1} (Table 2), a recovery of 13% of the activity present in the starting mitochondrial preparation and an increase of at least 50-fold over the activity observed with the detergent extract. The malonyl-CoA sensitivity of the reactivated CPTI was 80%. These results strongly suggest that detergent inactivation of CPTI is at least partially reversible.

Biochemical Characteristics of Yeast-Expressed CPTII. Detergent solubilization of mitochondria isolated from yeast cells expressing CPTII showed a high level of CPT activity that was highly resistant to inhibition by malonyl-CoA (Table 2). Furthermore, isolated mitochondria from the cells expressing CPTII exhibited only 40% of the CPT activity observed in detergent-solubilized mitochondria, indicating that the remaining 60% was latent and could only be released by detergent solubilization. The maximum level of CPTII activity observed without albumin in the reaction mixture was 35 nmol min^{-1} (mg of protein)$^{-1}$. CPTII activity in both isolated mitochondria and the detergent-solubilized mitochondria was much less sensitive to malonyl-CoA than CPTI (Table 2). The significance of the low malonyl-CoA sensitivity of the yeast-expressed CPTII is not known, but this is similar to the residual malonyl-CoA sensitivity observed in detergent-solubilized RLM (Woldegiorgis et al., 1992).

Detergent-solubilized yeast mitochondria containing CPTII exhibited saturation kinetics with respect to carnitine as shown in Figure 4C. For *P. pastoris*-expressed CPTII, the calculated K_m value for carnitine was 105 μM. The V_{max} for carnitine was 16 μM. As with CPTI, the detergent-solubilized yeast-expressed CPTII showed abnormal non-saturation kinetics with respect to palmitoyl-CoA (Figure 4D).

DISCUSSION

We report the high-level expression of active CPTI from rat liver in the yeast *P. pastoris*. The yeast-expressed CPTI is sensitive to inhibition by malonyl-CoA and detergent solubilization, unique properties predicted for RLM CPTI (Bremer, 1981; Bremer et al., 1985; McGarry et al., 1989). This is the first report of: (1) high-level synthesis of active CPTI in an expression system devoid of endogenous CPT activity and (2) the kinetic characteristics of CPTI in the absence of CPTII. The level of expression of CPTI in *P. pastoris* at 7.8 nmol of palmitoylcarnitine formed min^{-1} (mg of mitochondrial protein)$^{-1}$ is ~2−3 times that of the malonyl-CoA-sensitive CPT activity observed in RLM

Table 2: Characteristics of CPT Activities in Yeast Strains Expressing Rat Liver Mitochondrial CPTI or CPTII[a]

	intact mitochondria			detergent-solubilized mitochondria			reconstituted proteoliposomes		
	malonyl-CoA			malonyl-CoA			malonyl-CoA		
strain	−	+	% inhibition	−	+	% inhibition	−	+	% inhibition
WWG0 (control)	0	0	−	−	−	−	−	−	−
WWG1-7 (CPTI)	7.81 ± 0.6	0.56 ± 0.05	92.8	0.02	0	−	1.00 ± 0.1	0.20 ± 0.03	80
WWG2-1 (CPTII)	2.84 ± 0.4	1.87 ± 0.1	34.2	6.93 ± 0.34	5.49 ± 0.2	20.8	−	−	−

[a] Mitochondria were isolated from the yeast strains, separately expressing CPTI or CPTII, and solubilized in 5% Triton X-100 (10 mg of protein/mL), as described under Experimental Procedures. For CPTI, the detergent-solubilized mitochondria were reconstituted into liposomes by removal of the detergent in the presence of phospholipids on a Biobeads SM-2 column (see Experimental Procedures for details). Mitochondria, detergent-solubilized mitochondria, and reconstituted proteoliposomes were then assayed for CPT activities in the presence or absence of malonyl-CoA. The % inhibition of CPT in the presence of 100 μM malonyl-CoA is indicated. CPT activity is expressed in nmol min^{-1} (mg of protein)$^{-1}$. Results are an average of three separate experiments (mean ± SD).

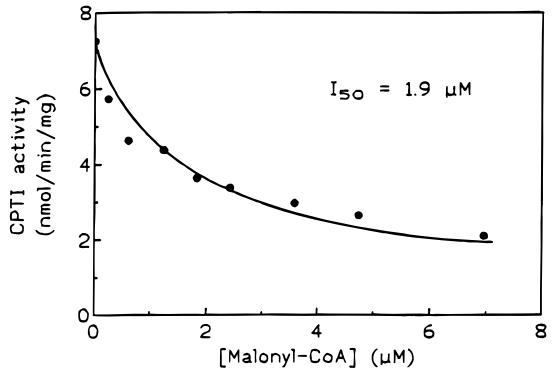

FIGURE 3: Effect of malonyl-CoA concentration on yeast-expressed CPTI activity. Isolated mitochondria (150 μg) from the yeast strain expressing CPTI were assayed for CPT activity in the presence of increasing concentrations of malonyl-CoA as described under Experimental Procedures.

(McGarry et al., 1983; Bremer et al., 1985) or in COS cell-expressing CPTI (Esser et al., 1993a) and is at least 1000-fold higher than the activity reported for RLM CPTI expression in the yeast *S. cerevisiae* (Brown et al., 1994b). The *P. pastoris*-expressed CPTI is located in the mitochondria as shown by differential centrifugation and sucrose density gradient fractionations. Thus, *P. pastoris* appears to be an excellent model system to investigate RLM CPTI.

CPTI is inactivated when removed from its membrane environment by detergents. Thus, rat liver, heart, and skeletal muscle mitochondria appeared to contain malonyl-CoA binding proteins that are distinct from CPT activity (Woldegiorgis et al., 1985; Lund & Woldegiorgis, 1987; Chung et al., 1992; Saggerson et al., 1992), because the purified proteins, all ~88 kDa in size, had no CPT activity. When we purified malonyl-CoA binding proteins from rat liver or heart mitochondria (Chung et al., 1992; Woldegiorgis et al., 1992) and reconstituted the malonyl-CoA binding proteins with detergent-solubilized malonyl-CoA-insensitive CPTII in the presence of phospholipids, we restored malonyl-CoA-sensitive CPT activity. Total CPT activity in the proteoliposomes was more than twice the initial activity with ~50% of the activity inhibited by malonyl-CoA. The data presented herein strongly suggest that CPTI was reactivated in these studies (Chung et al., 1992; Woldegiorgis et al., 1992). The other CPT activity was insensitive to malonyl-CoA, which likely represented the added CPTII. Recently, using a modified reconstitution procedure, we succeeded in restoring malonyl-CoA-sensitive CPT activity to Triton X-100-solubilized RLM by removal of the detergent in the presence of phospholipids, indicating reactivation of detergent-inactivated CPTI (unpublished observations). Detergent-inactivated CPTI expressed in yeast was also reactivated by detergent removal in the presence of phospholipids, similar to that observed with RLM. However, the reconstituted yeast-expressed CPT activity is more malonyl-CoA-sensitive than the reconstituted rat liver or heart mitochondrial CPTI. This is most likely due to the fact that, unlike mammalian mitochondria that contain a mixture of both CPTs, the yeast mitochondria contain only CPTI. Thus, removal of CPTI from its membrane lipid environment clearly inactivates this enzyme, and reconstitution via detergent removal in the presence of phospholipids reactivates a portion of the inactive CPTI, results that suggest CPTI is active only in a membrane environment. This is the first demonstration of direct reactivation of malonyl-CoA-sensitive CPTI activity from solubilized materials from any source. Until now, CPTI was presumed to be irreversibly inactivated by detergents and, hence, not recoverable as an active enzyme (McGarry et al., 1989). This report establishes for the first time that detergent inactivation of CPTI is reversible. This improved reconstitution procedure is faster and provides proteoliposomes of the same activity as those reported by us using a different method (Chung et al., 1992; Woldegiorgis et al., 1992). The ability to reconstitute yeast-expressed CPTI is critical to its purification and further studies on the structure and function of this important enzyme.

We also expressed rat liver CPTII in *P. pastoris*. Yeast-expressed CPTII is far less sensitive to malonyl-CoA inhibition than CPTI, and detergent solubilization of the mitochondrial membrane results in maximum release of CPT activity indicating the stability of the enzyme in detergents (Woldegiorgis et al., 1985; Bieber, 1988; McGarry et al., 1989; Brown et al., 1994a). Furthermore, exclusion of albumin from the assay mixture produced increased CPTII activity in contrast to reduced CPTI activity levels observed in the absence of albumin (unpublished observations), probably due to the detergent effect of palmitoyl-CoA that is countered by binding to albumin. About 60% of the *P. pastoris*-expressed CPTII activity was latent and could only be released by detergent solubilization of the mitochondrial membrane (Brown et al., 1994a).

Two models have been proposed to explain the loss of malonyl-CoA-sensitive CPT activity upon detergent solubilization of the mitochondrial membrane. Our group (Chung et al., 1992; Woldegiorgis et al., 1992) and others (Saggerson et al., 1992) proposed that CPTI and CPTII are either identical enzymes or isoforms and that a separate malonyl-CoA binding protein exists that interacts with CPTI and

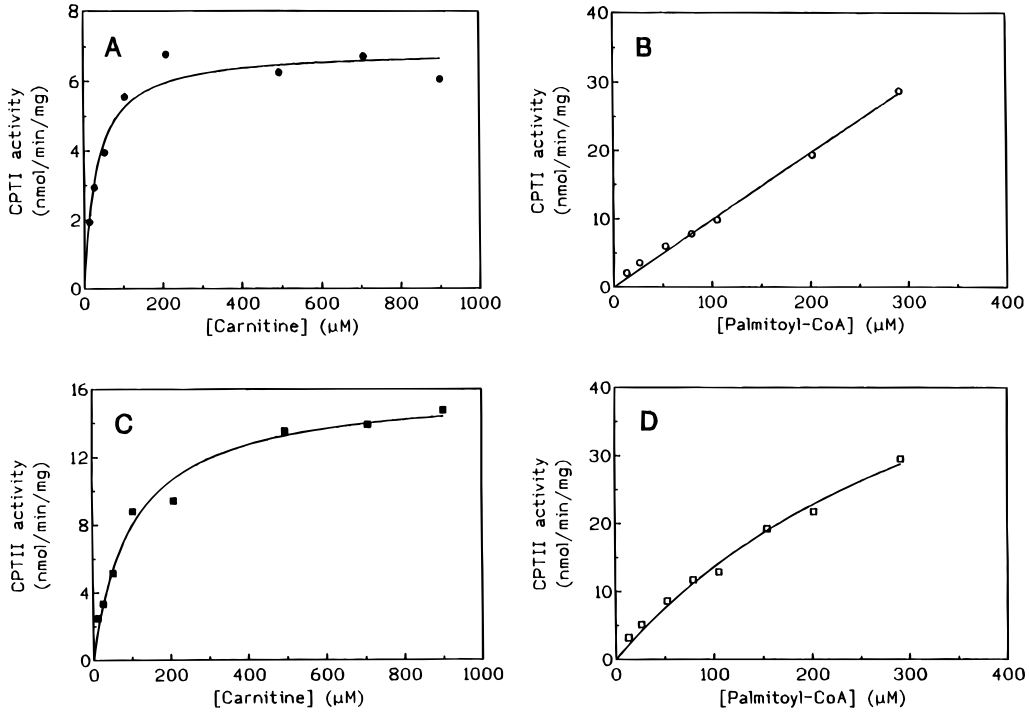

FIGURE 4: Effect of carnitine and palmitoyl-CoA concentrations on yeast-expressed CPTI and CPTII activities. Isolated mitochondria (150 μg) from yeast strains expressing CPTI and detergent-solubilized mitochondria (150 μg) from the yeast strain expressing CPTII were assayed for CPT activity in the presence of increasing concentrations of carnitine and palmitoyl-CoA, as described under Experimental Procedures. The figure shows the resulting dose−response curves for CPTI, carnitine (A); palmitoyl-CoA (B); CPTII, carnitine (C); and palmitoyl-CoA (D).

confers malonyl-CoA sensitivity to CPTI or CPTII. According to this model, detergents dissociate CPTI from its regulatory component, rendering it insensitive to malonyl-CoA inhibition. As evidence for this model, we were able to restore malonyl-CoA sensitivity to CPT in solubilized rat liver (Woldegiorgis et al., 1992) and heart mitochondria (Chung et al., 1992) by reconstitution of soluble malonyl-CoA-insensitive CPT with a purified malonyl-CoA binding protein devoid of CPT activity. The terms malonyl-CoA binding protein/regulatory component and/or inactive CPTI have been used in both models in reference to the same protein which binds malonyl-CoA and other inhibitors of the CPT system.

The alternative model proposed to explain the loss of malonyl-CoA-sensitive CPT activity upon detergent solubilization is that CPTI and CPTII are two distinct catalytically active proteins with the catalytic activity and malonyl-CoA sensitivity residing in a single polypeptide, CPTI (McGarry et al., 1989; McGarry, 1995). In this model, CPTI loses enzyme activity but not malonyl-CoA binding when removed from its membrane environment by detergents. Our data on the reconstitution of malonyl-CoA-sensitive CPT activity are then interpreted as reactivation of CPTI in this model. Previous reports on the expression of a CPTI cDNA were performed either in COS cells (Esser et al., 1993a), which have endogenous CPT activity, or in the yeast *S. cerevisiae* (Brown et al., 1994b), where the expression levels were too low to study the enzyme and reach a definitive conclusion as to whether CPTI is a distinct catalytically active malonyl-CoA-sensitive protein or a regulatory protein that confers malonyl-CoA sensitivity to the outer enzyme or CPTII. Results presented here clearly show that *P. pastoris*-expressed CPTI is malonyl-CoA-sensitive, detergent-inactivated, and reconstitutable.

Both yeast-expressed CPTI and CPTII exhibited saturation kinetics with respect to carnitine. With respect to the second substrate, palmitoyl-CoA, both yeast-expressed enzymes showed non-Michaelis−Menten saturation kinetics. This unusual kinetic behavior is also seen with the CPTs from RLM (McGarry et al., 1983; Brown et al., 1994a). Relative to CPTII, the nonsaturable kinetic behavior is more pronounced with yeast-expressed CPTI. This may be due to the fact that CPTI kinetic parameters were determined with intact mitochondria, whereas CPTII kinetic parameters were determined with a detergent-solubilized system. The reason for this unusual kinetic behavior is not known. Since the expressed enzymes exhibit saturable kinetics with respect to carnitine, and the immunoblots indicate that the molecular masses of the yeast-expressed CPTI and CPTII are identical to the molecular species detected in RLM (Woeltje et al., 1990a,b; Esser et al., 1993a), the nonsaturable kinetic behavior does not appear to be due to specific or nonspecific processing of the enzymes in the yeast expression system. The assay mixture contained fatty acid-free albumin to minimize the detergent effect of the substrate, palmitoyl-CoA; hence, the concentration of free palmitoyl-CoA available as substrate for the enzymes is unknown. Another possibility is that a yeast mitochondrial membrane lipid environment is different from RLM, or that one or more protein−protein interactions that occur in RLM do not occur in yeast.

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