Expression of precursor and mature carnitine palmitoyltransferase II in *Escherichia coli* and *in vitro*: differential behaviour of rat and human isoforms

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cDNAs corresponding to the precursor and mature forms of rat carnitine palmitoyltransferase II (CPT II) were found to be readily expressed in *Escherichia coli*. In both cases, catalytically active immunoreactive protein was produced and became largely membrane-associated. The precursor form of the enzyme was not proteolytically processed. Removal of 126 bp from the 5' end of the cDNA coding region allowed expression of a truncated CPT II (lacking the N-terminal 17 residues of the mature protein), but this product was inactive. cDNAs encoding the precursor and mature forms of human CPT II resisted direct expression in *E. coli*. However, the impediment was overcome when the latter cDNA was ligated in-frame 3' to sequence encoding a glutathione S-transferase. This construct yielded abundant quantities of the

corresponding fusion protein, a portion of which was soluble and catalytically active. *In vitro* transcription and translation of the various cDNAs established that the lower mobility on SDS/PAGE of rat CPT II compared with its human counterpart (despite their identical numbers of amino acids) is an intrinsic property of the primary sequences of the proteins themselves. Also, the human cDNA was found to contain an artifactual termination signal for T3 RNA polymerase that could be bypassed by the T7 polymerase. Thus rat CPT II can be expressed in active form in *E. coli* with characteristics similar to those of the enzyme in mitochondria, opening the way to future location of active sites within the molecule. An alternative expression system will be needed for similar studies on human CPT II.

INTRODUCTION

Carnitine palmitoyltransferases I and II (CPT I and II), in conjunction with a specific translocase, effect the transport of long-chain fatty acids into the mitochondrial matrix for β -oxidation. Because of its pivotal regulatory role in fuel metabolism [1,2], the CPT system is viewed as a potential target site for pharmacological intervention in disease states characterized by excessive rates of fatty acid oxidation [3,4]. Although the primary structures of rat and human CPT II [5–7] and rat liver CPT I [8] have recently been determined, they do not in themselves define the active sites of the molecules. To obtain such information, which would be valuable in designing potential inhibitors, we plan to utilize the techniques of site-directed mutagenesis followed by expression of CPT cDNAs in bacteria or other host cells. A necessary first step is to demonstrate that bacteria can synthesize CPT enzymes in a form similar to those produced in the mammalian organism. This was the primary goal of the present work. A second was to seek an explanation for the apparently larger molecular mass of native rat versus human CPT II on SDS/PAGE [9], despite the fact that both proteins are initially synthesized as 658-amino-acid precursors from which a 25-residue leader peptide is cleaved upon translocation into the mitochondrion [6,10].

In the studies outlined below we show that rat CPT II is readily expressed in *E. coli* in an active, membrane-associated form. Also established is that the differing electrophoretic mobilities of the rat and human isoforms results from aberrant

behaviour of one or other of the proteins on SDS gels. Finally, some unusual functional characteristics of the human CPT II cDNA, as regards its expression both in *E. coli* and *in vitro*, are documented.

EXPERIMENTAL

Construction of rat CPT II expression plasmids

Two major plasmid types were prepared. pBluescript II-based plasmids were used to make constructs suitable for *in vitro* transcription, while pKK223-3-based plasmids were employed for expression in *E. coli*. The final constructs, illustrated in Figure 1, are designated pB- pKK- followed by R or H to indicate the animal species from which the DNA was isolated. The position of the initiator methionine codon relative to the full-length precursor form of the enzyme completes identification of the construct. In the text, nucleotides are numbered with the first base of sequence encoding the full-length enzyme designated as +1.

Isolation of a full-length cDNA clone encoding the precursor form of rat CPT II has been described (ref. [5]; that construct, pBKS-CPT II.4, is here referred to as pB-RMet1). The PCR was employed to amplify the coding region of plasmid pB-RMet1 using a 5' oligonucleotide that included an *EcoRI* restriction site (CCCGAATTCAAAATGATGCCGCGCCTGCTGTT TCG) and a 3' oligonucleotide containing the original termination codon, an additional termination codon and a *HindIII* site (GGGAAGCTTCATGACTAAGTTTTGATGGCTTTGCC-

Abbreviations used: CPT, carnitine palmitoyltransferase (EC 2.3.1.21); GDH, glutamate dehydrogenase (EC 1.4.1.4); GST, glutathione S-transferase (EC 2.5.1.18); IPTG, isopropyl β -D-thiogalactopyranoside; OG, octyl glucoside; PMSF, phenylmethanesulphonyl fluoride.

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Figure 1 CPT II expression plasmids

Single lines, CPT II coding regions; broken lines, CPT II untranslated regions; double lines, vectors; GST, GST coding region; T3P, T7P and tacP, promoters; B, E, H, Nco, Not, P, S and X, restriction sites for *Bam*HI, *Eco*RI, *Hind*III, *Nco*I, *Not*I, *Pst*I, *Sal*I and *Xho*I respectively; Met1 and Stop, beginning and end respectively of the rat CPT II coding region in pB-RMet1. *Bam*HI sites are shown for pGST/HMet25 only; *Pst*I, *Xho*I and *Nco*I sites are shown for the pKK-HMet1 coding sequence only. Only correctly oriented promoters are indicated. The diagram is not to scale.

TTC). The product was cut with *Eco*RI and *Hind*III and ligated into *Eco*RI/*Hind*III-cut pKK223-3 to give pKK-RMet1. This placed the rat CPT II coding region downstream of the powerful *tac* promoter and positioned the initiating Met-1 codon at an appropriate distance from the plasmid's ribosome-binding site.

In a second PCR reaction, a portion of pB-RMet1 was amplified using a 5' oligonucleotide containing an *Eco*RI site and an artificial methionine codon before coding base pairs 76–97 (CCCGAATTCAAAATGAGTGCTGTCTCGGGGCCGGA-CG), and an internal 3' oligonucleotide (CTCTGTGCGGCCG-TGCTTGA). The product was cut with *Eco*RI and *Xho*I, and the resulting fragment ligated into *Eco*RI/*Xho*I-cut pKK-RMet1. The product, pKK-RMet25, contained a novel initiator methionine codon followed by the coding region for the mature form of rat CPT II (amino acids 26–658).

By a similar process, part of pB-RMet1 was PCR-amplified using a 5' oligonucleotide containing an *Eco*RI site plus coding base pairs 127–147 (CCCGAATTCAAAATGCACTACCAGG-ACAGCCTG), and the internal 3' oligonucleotide described above. This product was cut with *Eco*RI and *Xho*I and ligated into *Eco*RI/*Xho*I-cut pKK-RMet1 to give pKK-RMet43, de-

signed to express a truncated form of rat CPT II beginning at the naturally occurring Met-43.

The coding region of pKK-RMet25 was excised with *Eco*RI and *Hind*III and ligated into *Eco*RI/*Hind*III-cut pBluescript II KS(+) to yield pB-RMet25.

Construction of human CPT II expression plasmids

Human foetal liver cDNA was generously provided by G. McMahon and L. McCarthy (Sandoz Research Institute). Two oligonucleotides within the coding region were used to amplify a portion of the CPT II sequence by PCR (5' oligonucleotide, GAACTGCATGAGCAGCTGGTTGCTC; 3' oligonucleotide, ACTTTTGATGGATTTCCTTCTAAGGC). The product was cut with *Xho*I and *Nco*I to give a DNA fragment stretching from base 368 to 1614 of the coding region.

Partial cDNA clones, in vector M13, covering most of the human CPT II coding region have been isolated previously [11]. Two of these, together with the above PCR-derived fragment and a partial genomic clone covering the extreme 5' end of the

coding region, were used to complete a full-length human CPT II cDNA clone, as described below.

One partial cDNA clone (including the 3' end of the coding region) was PCR-amplified using a 5' oligonucleotide within its M13 vector (GTTTTCCAGTCACGAC) plus a 3' oligonucleotide including the original termination codon, an additional termination codon and a *Hind*III restriction site (GGGAAGCTTGGATCCTAAAGTTAACTTTTGATGGA-TTT). The product was cut with *Nco*I and *Hind*III to yield a DNA fragment covering base 1614 to the 3' end of the CPT II coding region (base pair 1974). The base pair 368–1614 and 1614–1974 fragments were ligated together and the resulting concatamers cut with *Xho*I and *Hind*III to give three products. The mid-sized product, encoding base pairs 368–1974, was purified and ligated into *Xho*I/*Hind*III-cut pBluescript II KS(+). The plasmid was grown and the *Xho*I/*Hind*III insert was reisolated and treated with *Eco*RI methylase to protect the internal *Eco*RI restriction site.

A second partial cDNA clone (including the 5' end of sequence encoding the mature protein) was PCR-amplified using a 5' oligonucleotide that included an *Eco*RI site, an artificial methionine codon plus coding base pairs 76–96 (CCCGAATTCAA-AATGAGCGCCGGCTCCGGGCCCGGC), and an internal 3' oligonucleotide (AGGGTTCAAGTGGAACTTCTGG). The product was cut with *Eco*RI and *Xho*I to yield a fragment covering base pairs 76–368 of the coding region. This, and the fragment covering base pairs 368–1974 (above), were ligated and the concatamers produced were cut with *Eco*RI and *Hind*III. The mid-sized product was purified to yield a DNA fragment covering base pairs 76–1974, with a novel methionine codon immediately preceding the DNA sequence coding for the mature form of human CPT II (amino acids 26–658). This was ligated into pBluescript II SK(+) and pKK223-3 (cut with *Eco*RI and *Hind*III) to give plasmids pB-HMet25 and pKK-HMet25 respectively.

A partial human genomic clone (including sequence encoding the leader peptide) was PCR-amplified using a 5' oligonucleotide containing an *Eco*RI site (CCCGAATTCAAATGGTGCCCGCCTGCTGCTG) and an internal 3' oligonucleotide (GGCAGCCTGGGCAGGCTGTCTGGTAGTGCATGGT). The product was cut with *Eco*RI and *Pst*I to form a fragment covering base pairs 1–103 of the coding region. This was ligated with the *Eco*RI-methylated *Pst*I/*Hind*III fragment of pKK-HMet25 (base pairs 103–1974). The resulting concatamers were cut with *Eco*RI and *Hind*III and the mid-sized fragment encoding base pairs 1–1974 of the CPT II coding region was purified and ligated into *Eco*RI/*Hind*III-cut pBluescript II SK(+) and pKK223-3 to give plasmids pB-HMet1 and pKK-HMet1 respectively.

*Bam*HI and *Sal*I, which cut within the polylinker, were used to liberate the inserts of pB-HMet1 and pB-HMet25. These were then ligated into pBluescript II KS(+) to give pB-HMet 1-T7 and pB-HMet25-T7 respectively. *In vitro* transcription of these two plasmids was achieved using T7 RNA polymerase, whereas transcription from all other pBluescript plasmids normally employed T3 RNA polymerase.

A further plasmid was constructed to express the mature form of human CPT II in *E. coli* as a fusion protein. The entire coding region of pKK-HMet25 was PCR-amplified using 5' and 3' oligonucleotides containing *Bam*HI restriction sites (5' oligonucleotide, CAGGATCCTCAGCGCCGGCTCCGGG; 3' oligonucleotide, GGGAAGCTTGGATCCTAAAGTTAACTTTTGATGGATTT). The *Bam*HI-cut product was ligated into *Bam*HI-cut pGEX-3X to give pGST/HMet25. The coding region for mature human CPT II was thus placed, in-frame, immediately

3' to the sequence encoding ~27 kDa of a schistosomal glutathione S-transferase (GST).

The fidelity of all PCR reactions and ligations was confirmed by DNA sequencing.

Bacterial culture and fractionation

The host strain was *E. coli* JM109 throughout. Cells were grown at 37 °C in shaking flasks containing LB broth (10 g of tryptone, 5 g of yeast extract and 5 g of NaCl/litre), including 50 µg/ml ampicillin when a plasmid was present. Overnight cultures (3 ml) were used to inoculate 50 or 100 ml of LB ($\pm$ ampicillin) to an initial A_{650} of 0.1, after which the cultures were grown to an A_{650} of 0.4–0.5. In most cases, isopropyl β -D-thiogalactopyranoside (IPTG) was then added to a concentration of 1 mM and the culture was continued for various periods of time. IPTG was omitted for expression of the GST/HMet25 fusion protein.

A volume of cell culture equivalent to 5 absorbance units (rat constructs) or 20 absorbance units (human constructs, to compensate in part for the lower specific activity) was centrifuged at 2900 g for 10 min at room temperature, after which the cell pellet was resuspended in 850 µl of 10 mM Tris/HCl, pH 7.2, and transferred to a 1.5 ml microcentrifuge tube. For protein assay, 50 µl was removed. To the remainder was added 200 µl of lysozyme (5 mg/ml in 10 mM EDTA and 10 mM Tris/HCl, pH 7.2) and the mixture was rocked for 25 min at room temperature.

For time course experiments, 50 µl of 20% (w/v) octyl glucoside (OG) was added and the mixture was kept on ice for 30 min with frequent mixing prior to assays.

For subcellular localization experiments, two samples from each culture were prepared as described above, up to and including lysozyme treatment. They were then frozen twice in liquid N₂ and thawed. One sample was centrifuged for 5 min in a Microfuge. The supernatant was collected and received 50 µl of 20% (w/v) OG, while the pellet was resuspended in 1 ml of 10 mM Tris/HCl, pH 7.2, and 1% (w/v) OG (200 µl of each pellet and supernatant fraction was stored at –20 °C and used for Western blots within 2 weeks). The second sample received 50 µl of 20% (w/v) OG and was left on ice for 30 min. Half (500 µl) was then centrifuged as above, the supernatant was collected and the pellet resuspended in 500 µl of 50 mM Tris/HCl, pH 7.2, and 1% OG (w/v). At the time of assay, all samples had been exposed to OG at 0 °C for at least 30 min.

Enzyme assays

CPT II activity was assayed as previously described [12], using DL-[methyl-¹⁴C]carnitine or L-[methyl-³H]carnitine. Glutamate dehydrogenase (GDH) was measured in a total volume of 1 ml containing 50 mM Tris/HCl, pH 8.0, 2.5 mM EDTA, 1 mM ADP, 100 mM NH₄Cl, 0.2 mM NADPH and 7 mM α -oxoglutarate. The reaction was started by addition of the α -oxoglutarate and monitored by the decrease in absorbance at 340 nm. Protein measurement was by the method of Lowry et al. [13].

Purification of pKK-RMet1 and pKK-RMet25 products

After inoculation to an A_{650} of 0.1 and growth to an A_{650} of 0.4–0.5, 1-litre cultures were made 1 mM with respect to IPTG and grown for a further 4 h. Cells were harvested by centrifugation at 5000 g for 15 min, frozen as cell pellets in liquid N₂ and stored at –20 °C. Five such pellets were resuspended in 200 ml of 10 mM Tris/HCl, pH 7.5, and 1 mM phenylmethane-

sulphonyl fluoride (PMSF), to which was then added 50 ml of lysozyme (5 mg/ml) in the same buffer. The mixture was stirred for 25 min at room temperature and then frozen in liquid N_2 . After thawing, 2.5 ml of 100 mM PMSF (in propan-2-ol) and 12.5 ml of 20% (v/v) Tween-20 were added, and the mixture was stirred for 60 min on ice before centrifugation at 100 000 g for 30 min. Although CPT II displays greater activity in OG, Tween-20 was chosen as a vehicle for purification since it had been used previously for immunopurification of the enzyme [10]. The supernatant was kept at 4 °C and made 30% with respect to $(NH_4)_2SO_4$, followed by centrifugation at 100 000 g for 20 min at 4 °C. The supernatant was decanted and its $(NH_4)_2SO_4$ concentration was raised to 50%. After further centrifugation, the pellet was resuspended in 18 ml of 10 mM Tris/HCl, pH 7.5, and 1% Tween-20. DNAase was added to 2 μ g/ml and the mixture was rocked for 90 min at room temperature, followed by centrifugation at 100 000 g for 30 min. The supernatant was collected and adjusted to pH 7.5 with 5 M NaOH. Portions (0.5 ml) were applied to an anti-(rat CPT II) immunoaffinity column for further purification as previously described [10]. Each subsequent batch of product was resuspended in 100 μ l of SDS/PAGE sample buffer (62.5 mM Tris/HCl, pH 6.8, 6.25% glycerol, 5% SDS and 5% β -mercaptoethanol) and heated at 60 °C for 15 min. Aliquots were then used for SDS/PAGE and N-terminal sequencing [10]. A polyclonal antibody raised against rat liver CPT II [14] was employed for all immunoblots and immunopurification.

In vitro transcription and translation

pBluescript plasmids were linearized downstream of the coding region with *Not*I (pB-RMet1) or *Sal*I (others), except where otherwise stated. Subsequent transcription and translation were as previously described, using [3H]leucine in a rabbit reticulocyte lysate system [10]. In addition, portions of the transcripts were run on 1% agarose gels and transferred to nylon membranes for Northern blot analysis using a 1.4 kb ^{32}P -labelled probe (the *Xho*I/*Bam*HI fragment of pB-RMet1). ^{32}P -labelled phage λ /*Hind*III fragments were run as standards.

Materials

Sources of most of the materials have been given previously [9,10,15]. Others were as follows: IPTG, ovalbumin (Sigma, St. Louis, MO, U.S.A.); *Eco*RI methylase (Promega, Madison, WI, U.S.A.); T3 and T7 RNA polymerases, pBluescript II plasmids (Stratagene, La-Jolla, CA, U.S.A.); lysozyme, DNAase (Boehringer Mannheim, Indianapolis, IN, U.S.A.); *Sal*I, plasmids pGEX-3X and pKK223-3 (Pharmacia LKB, Piscataway, NJ, U.S.A.); other restriction enzymes, T4 DNA ligase (New England Biolabs, Beverly, MA, U.S.A.); L-[methyl- 3H]carnitine (Amersham, Arlington Heights, IL, U.S.A.).

RESULTS AND DISCUSSION

Expression of eukaryotic enzymes in *E. coli* is a useful tool for exploring their functional domains. In the present study we have begun to use this approach with the mitochondrial inner membrane protein CPT II. Our first priority was to determine if the bacteria would produce the foreign protein in an active form and in sufficient quantities for subsequent analysis. It also had to be ascertained whether the leader sequence of CPT II would help or hinder expression of the enzyme.

Plasmids pKK-RMet1 and pKK-RMet25 were designed to express the precursor and mature forms of rat CPT II respectively

in bacteria (Figure 1). Figure 2 shows that *E. coli* JM109 cells containing these plasmids expressed CPT II activity; non-transformed cells did not (results not shown). Expression of the precursor form of the enzyme yielded approximately 2-fold greater activity than the mature form at each time point. When IPTG, an inducer of the *tac* promoter, was not used, enzyme activity was still seen, but at about half the levels found in its presence (results not shown). Maximal activities were reached between 4 and 8 h after induction. A third plasmid, pKK-RMet43, was used to express an N-terminally truncated form of rat CPT II beginning with the naturally occurring Met43. *E. coli* containing this construct displayed no measurable CPT activity (Figure 2). Cells containing plasmids pKK-HMet1 and pKK-HMet25, intended to express precursor and mature forms respectively of the human enzyme, failed to show more than an occasional trace of enzyme activity. These plasmids were in every respect identical with the corresponding rat constructs, except for the coding regions themselves (see Figure 1). To confirm that failure of the cells to express active human CPT II was not due to an error having arisen in the sequence of the pKK223-3 expression vector itself, the coding region of pKK-HMet25 was excised and replaced with that of pKK-RMet25. The resulting plasmid expressed abundant CPT activity (results not shown), indicating that the lack of response with the human constructs (Figure 2) stemmed from a peculiarity of the human CPT II sequence itself. It is known that codon usage can radically alter the efficiency of expression of foreign proteins in *E. coli* [16,17] and that the problem with poor expression can often be overcome by the use of a fusion protein construct [18]. Accordingly, the sequence for mature human CPT II was placed downstream of a sequence encoding 27 kDa of a GST (plasmid pGST/HMet25). The fusion protein was readily expressed and, surprisingly, generated a small but consistently observed CPT activity (Figure 2). This was always more noticeable if the inducer, IPTG, was not present (results not shown). Such behaviour is characteristic

Figure 2 Time course of expression of CPT II activity in *E. coli*

Initial culture was as described in the Experimental section. When the cell density had reached an A_{650} of 0.4–0.5, IPTG was added to 1 mM (except for pGST/HMet25). Samples were collected at the indicated times for CPT assay. Results are expressed relative to total cellular protein.

Figure 3 Immunoblot of recombinant rat CPT II in *E. coli*

Culture conditions were as for Figure 2. Portions of the OG-solubilized cells from the 4 h time point were mixed with an equal volume of $2 \times$ SDS/PAGE sample buffer and boiled for 3 min. A 100 μ l portion of each was subjected to electrophoresis on an 8% gel for Western blot analysis. The arrow indicates the migration position of purified rat liver CPT II.

of the formation of inclusion bodies, where lowering the rate of foreign protein synthesis may lead to more efficient production of the protein in an active form [18]. Consequently, in all experiments with plasmid pGST/HMet25 shown here, IPTG was omitted.

Although cells containing pKK-RMet1 appeared to express a higher level of CPT activity than their counterparts carrying pKK-RMet25, it is possible that they simply produced more recombinant protein (which was not rigorously quantified here) and that little difference existed in the actual specific activities of the two products. In any event, it is evident that the presence of the mitochondrial targeting peptide does not impair, and perhaps even enhances, the catalytic activity of the recombinant enzyme. In absolute terms, the level achieved (sometimes as high as 2 nmol/min per mg of total cell protein) is quite impressive considering that under similar assay conditions our values for CPT II activity in isolated rat liver mitochondria are generally in the region of 5–6 nmol/min per mg of protein.

Western blot analysis of cell extracts using a polyclonal antibody raised against rat liver CPT II confirmed the presence of the rat protein in expressing cells (Figure 3, lanes 2 and 3). No reaction was observed in non-transformed cells (lane 1). However, both pKK-RMet1 and pKK-RMet25 gave rise to two immunoreactive bands, and in each case the larger, major band approximated to purified rat liver CPT II in mobility. The lower-molecular-mass band was present at every stage of cell growth and induction, suggesting that no precursor–product relationship existed between the reacting species. Plasmid pKK-RMet43 generated a single reacting band on Western blots that co-migrated with the smaller, minor products of pKK-RMet1 and pKK-RMet25. It is possible that the smaller protein produced from pKK-RMet1 and pKK-RMet25 represented low-level translation initiated from internal Met-43. The inactivity of the truncated protein product suggests that the N-terminal 17 amino

Figure 4 Immunoblot of recombinant human CPT II in *E. coli*

Samples were prepared and analysed as described in the legend to Figure 3.

acids of mature CPT II are essential for the production of active enzyme. Solubilized cells containing the human CPT II expression plasmids pKK-HMet1 and pKK-HMet25 failed to react with anti-(rat CPT II) antibody on Western blots (Figure 4, lanes 1 and 2). (The antibody has been previously shown to cross-react with the human protein [9].) However, pGST/HMet25 gave rise to a major reacting protein that was larger than CPT II itself (Figure 4, lane 3). A strong signal in the same position was seen using an anti-GST antibody (results not shown). Variable amounts of a lower-molecular-mass species were also detected by the antibody to CPT II and probably represented a degradation product (see Figures 4 and 6).

To determine if the expressed rat CPT II was being proteolytically processed by the bacteria, the products of pKK-RMet1 and pKK-RMet25 were purified and subjected to N-terminal sequencing. The results showed that the precursor form of the enzyme was not processed and retained its two N-terminal methionine residues. In contrast, the 'artificial' methionine residue introduced to initiate translation of the mature CPT II was removed, so that the final expressed product had a primary structure identical with that of the rat protein synthesized *in vivo*. SDS/PAGE analysis of the purified recombinant mature rat CPT II is illustrated in Figure 5. The immunoaffinity purification technique employed yielded two major protein species (the ovalbumin present at ~ 45 kDa was introduced as part of the purification protocol). The lower band did not react with the anti-(CPT II) antibody used in the purification (results not shown). It therefore did not correspond to the minor reacting species seen on Western blots (Figure 3). However, the N-terminal sequence obtained for the recombinant CPT II was heavily contaminated with the sequence Ala-Ala-Lys-Asp..., which is the known N-terminus of the *E. coli* heat-shock protein, GroEL. GroEL has a reported apparent molecular mass of ~ 65 kDa [19] and we believe that the lower-molecular-mass species that co-purified with CPT II is this protein.

To further investigate the validity of the expression system, the subcellular localization of the recombinant proteins was determined. NADPH-dependent GDH was employed as an

Figure 5 Partial purification of recombinant rat CPT II

Recombinant mature rat CPT II was isolated using an immunoaffinity column as described in the Experimental section. A 50 μ l sample of the product was subjected to SDS/PAGE on an 8% gel and stained with Coomassie Brilliant Blue. CPT II purified from rat liver was run in parallel. Bands representing recombinant CPT II, GroEL and ovalbumin (ovalb.) are indicated. Lane 1, CPT II standard; lane 2, eluate.

Figure 6 Subcellular localization of expressed CPT II isoforms

Culture and preparation of samples is described in the Experimental section. Samples (100 μ l) were applied to 8% polyacrylamide gels for Western blot analysis. P, pellet; S, supernatant.

Table 1 Subcellular localization of CPT II activity

Cells expressing functional CPT II were fractionated as described in the Experimental section. The activities of CPT and GDH found in the soluble fraction are expressed as percentages of the total activity recovered.

Construct	Soluble activity (% of total)			
	- OG		+ OG	
	CPT II	GDH	CPT II	GDH
pKK-RMet1	22	72	92	77
pKK-RMet25	40	75	92	73
pGST/HMet25	38	78	84	79

indicator of released soluble proteins. Expressing cells were lysed by freeze/thawing and the particulate and soluble material was separated by centrifugation before or after treatment with OG. A typical experiment is illustrated in Table 1. Even in the absence of OG the cell-lysing protocol released 70–80% of GDH activity. Under these conditions, however, most of the CPT II activity, regardless of the cDNA used, was associated with the particulate fraction. If the broken cells were lysed with OG prior to centrifugation, substantially more CPT activity was found to be soluble in all cases, reflecting the behaviour of CPT II in intact mitochondria [14]. The samples fractionated in the absence of OG were subjected to Western blot analysis (Figure 6). Each of the expressed rat proteins was detected in soluble and particulate fractions. In contrast, the vast majority of the fusion protein mass was apparently located in the particulate fraction of the cell (Figure 6, lanes 7 and 8), though much of the enzymically active material was found to be soluble (Table 1). Thus the specific activity of the soluble material might actually have been similar to that of the recombinant rat enzymes. The fact that *E. coli* produced the human fusion protein in abundance

suggests that the problem in expression of the pKK-HMet1 and -HMet25 constructs lay at the level of translation rather than in transcription, and was not related to the difficulties encountered during *in vitro* transcription using T3 RNA polymerase (see below).

A second objective of the present work was to investigate the paradoxical difference in the mobility of CPT II from rat and human liver during SDS/PAGE (the former runs with a higher apparent molecular mass [9], despite the fact that in the mature form both proteins are predicted to contain 633 amino acids and to differ in mass by only ~100 Da [11]). However, the inability of *E. coli* to express pKK-HMet1 or -HMet25 precluded direct comparison of the rat and human enzymes using the bacterial system. To circumvent this problem, we chose an *in vitro* transcription and translation approach. Initially, pBluescript plasmids were constructed to transcribe the precursor and mature forms of CPT II from both rat and human under the control of T3 RNA polymerase (Figure 1). Plasmids pB-RMet1 and pB-RMet25 were efficiently transcribed and translated in this system (Figures 7 and 8). Although the protein products were not easily distinguished on SDS/PAGE, a slight size difference could be discerned on high percentage gels (Figure 7, lanes 1 and 2). Transcription and translation of pB-HMet1 and pB-HMet25, however, yielded only a trace of the expected protein products, at about 70 kDa (Figure 7, lanes 5 and 6). The major protein species formed migrated with apparent molecular masses of only 45–50 kDa. To determine the basis for this phenomenon, we analysed the *in vitro* transcripts generated from each plasmid (Figure 8). Those derived from the two rat constructs were of the expected sizes (Figure 8, lanes 6 and 7), the difference between them reflecting the presence of some untranslated flanking sequence in plasmid pB-RMet1 (Figure 1). Transcription of the human constructs pB-HMet1 and pB-HMet25 yielded a trace of the expected RNA products (~2.2–2.3 kb; Figure 8, lanes 1 and 2), but in each case the major product was much smaller (~1.5–1.6 kb). To distinguish between the possibilities of aberrant RNA polymerase initiation and premature termination, plasmid pB-HMet25 was first linearized within the coding region using *Nco*I, and then subjected to *in vitro* transcription. The

Figure 7 Rat and human CPT II *in vitro* translation products

In vitro transcripts were translated in a rabbit reticulocyte lysate system containing [3 H]leucine. Portions of the products were applied to 13% polyacrylamide gels. Bromophenol Blue dye included in the samples eluted after 1 h, but electrophoresis was continued for another 1.5 h, a procedure necessary to differentiate the rat isoforms. Bands were detected by fluorography.

Figure 8 Northern blot analysis of CPT II *in vitro* transcripts

Transcripts were generated and analysed as described in the Experimental section.

major product was of the same size as had been seen with the full-length template (Figure 8, cf. lanes 1 and 3). However, the minor, higher-molecular-mass, transcript migrated faster than its counterpart generated from the full-length molecule. This demonstrates that the lower-molecular-mass transcripts in lanes 1 and 2 were the result of premature termination 5' to the *Nco*I site, as opposed to misplaced initiation. Examination of the human sequence [6,7,11] reveals an imperfect inverted repeat between base pairs 1253 and 1287 (1253 AGCTGACTGATGCC---GGCATCACAGCT 1287). If secondary structure in this region of the cDNA prevented progress of the T3 polymerase, the

predicted transcript and protein products would be of the sizes found here. Interestingly, this inverted repeat is poorly conserved in the rat sequence.

Transcription of pB-HMet25 in the reverse direction using T7 RNA polymerase yielded a full-length anti-sense product (results not shown). Thus it appeared that the T7 polymerase operating in the 3'→5' direction of the coding sequence could bypass whatever obstacle prevented progress of the T3 enzyme in the 5'→3' orientation. To exploit this observation, two further plasmids were constructed in which correct transcription of the human forms of CPT II could be directed by T7 RNA polymerase (pB-HMet1-T7 and pB-HMet25-T7). *In vitro* transcription and translation using these plasmids yielded RNA and protein products of the expected sizes (Figure 7, lanes 3 and 4; Figure 8, lanes 4 and 5).

Two important points emerged from these studies. First, the precursor and mature forms of rat CPT II migrate more slowly on SDS gels than their human equivalents. Secondly, the difference in mobility between the precursor and mature products is distinctly less in the case of rat CPT II. Clearly, the apparently larger size of the rat compared with the human enzyme is an artifact stemming from the difference in the peptide sequences themselves. We suspect that the rat protein behaves anomalously, in light of the fact that the CPT II enzymes from mouse, monkey and human liver all co-migrate on SDS gels [9]. This is a critical observation in light of the continued suggestion by Brady et al. [20] that our rat CPT II cDNA [5] is short at the 5' end of the coding region. We show here that our recombinant mature rat CPT II migrates on SDS gels identically with CPT II purified from rat liver. If the N-terminus of the native enzyme was as described by the above authors, the purified and recombinant proteins would have a calculated difference in molecular mass of ~4.3 kDa (38 amino acids), which should have been readily discernible. This, and additional evidence to be presented elsewhere, gives us confidence in the validity of our published sequence.

Finally, the fact that rat CPT II can be expressed in *E. coli* in an active form and with characteristics similar to those of the native enzyme should allow detailed mutagenic analysis of important regions of the molecule, and could aid in determination of its three-dimensional structure. An alternative expression vehicle will probably be needed in order to conduct similar studies on the human enzyme.

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