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Purification, characterization and partial amino acid sequences of carnitine palmitoyl-transferase from human liver

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Carnitine palmitoyl-transferase has been extracted with 0.5% Tween-20 from human liver homogenate and purified to homogeneity. The purified enzyme has a native M_r of 274 kDa. The subunit M_r is of 66 kDa, as shown by SDS-PAGE and immunoblots obtained with antibodies raised against human CPT. Purified CPT shows high affinity for palmitoyl-CoA and palmitoyl-carnitine and is not inhibited by malonyl-CoA. Seven tryptic peptides and the N-terminal of purified human CPT have been sequenced, and found homologous to rat CPT sequence. Both antibodies and peptide sequences are important tools for the investigation of the molecular basis of CPT deficiency in man.

Carnitine palmitoyl-transferase; Human liver; Purification; Amino acid sequence

1. INTRODUCTION

Carnitine palmitoyl-transferase (CPT) (EC 2.3.2.21) allows long chain acyl-CoAs to cross the mitochondrial membranes by catalyzing their reversible conjugation with L-carnitine [1]. One part of CPT activity is associated with the outer mitochondrial membrane while another pool of CPT activity is in close relationship with the inner mitochondrial membrane [2]. It is still controversial whether the two pools express the activity of a single protein, differently located in mitochondrial membranes, or of two different enzymes.

CPT has a key-function in the regulation of fatty acids oxidation. Accordingly, its activity is finely regulated by malonyl-CoA inhibition and by hormonal action, mostly of glucagon and insulin [3].

Inherited deficiency of CPT causes muscle weakness, cramps and myoglobinuria in adults [4] and coma, non-ketotic hypoglycemia, elevation of transaminases and free fatty acids in infants [5].

As a first step towards the understanding of the molecular basis of CPT deficiency in man we purified CPT from human liver, raised polyclonal antibodies against this enzyme and determined partial amino acid sequences.

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2. MATERIALS AND METHODS

2.1. *Enzyme assays*

CPT was assayed in the forward reaction by the 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) method [6]. The mixture contained 0.1 mM palmitoyl-CoA, 0.2 mM (DTNB), 0.1 M Tris-HCl (pH 8), 0.5 mM EDTA, 0.1% Tween-20. The reaction was started by adding 1 mM L-carnitine. One unit of enzyme activity corresponds to one μmol of palmitoyl-carnitine/min/mg. For the determination of pH optimum, the activity was assayed using 0.1 M potassium phosphate/Tris-glycine buffer, adjusted to the desired pH.

CPT activity was also assayed by following the formation of palmitoyl-CoA from CoASH and palmitoyl-carnitine (backward reaction) at 23 nm at 25°C [7]. The reaction mixture contained 91 mM Tris-HCl (pH 7.6), 0.19 mM CoASH and 5.5 mM palmitoyl-carnitine. In order to determine if human CPT is inhibited by malonyl-CoA the activity was assayed by the DTNB method in the presence of 0.1 mM octanoyl-CoA or palmitoyl-CoA and 0–0.1 mM malonyl-CoA. The reaction was started by adding the enzyme. Since rat CPT inhibition by malonyl-CoA is abolished by Tween-20 [8], such detergent was not present in the reaction buffer. The assay described by Solbert [9] was also used to check malonyl-CoA inhibition. The mixture contained 0.45 ml of 0.11 M Tris-HCl (pH 7.8), 5.6 mM GSH and 0.13 mM palmitoyl-CoA; the concentration of malonyl-CoA ranged from 0–0.1 mM. The reaction was started by 100 μl of 6 mM L-[^{3}H]carnitine, carried out at 30°C for 5 min and stopped with 1 ml isobutanol. One ml of saturated ammonium sulphate was added and the solution stirred. After centrifugation, 700–800 μl of supernatant were collected, the pellet washed with 1 ml of saturated ammonium sulphate and centrifuged [9]. The amount of radioactivity was determined in 500 μl of the combined supernatants.

Protein concentrations were estimated using bicinchoninic acid [10], according to the manufacturers' instructions (Pierce).

2.2. *Purification and properties of human CPT*

Normal human liver obtained 20–24 h after death was stored at −80°C. In a typical experiment 91 g of liver were homogenized in 350 ml of 20 mM Tris-HCl (pH 9.5), 5 mM EDTA, 0.5 mM phenylmethane sulphonyl fluoride, 0.5 mM phenantroline (buffer A). The suspension was centrifuged at 40 000 × g for 30 min. The pellet obtained was resuspended by sonication in buffer A plus 0.5% Tween-20, and centrifuged at 40 000 × g for 30 min. The supernatant was diluted three-fold with deionized water and applied to a DEAE-

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Table I

Purification steps of CPT from human liver

Step	Activity (μmol)	Proteins (mg)	Specific activity (μmol/mg)	Purification (-fold)	Yield (%)
1. Pellet-extract	49.0	6473.0	0.007	-	-
2. DEAE Seph. CL-6B	22.5	1720.0	0.013	1.9	45.9
3. CM-Sepharose	21.6	1200.0	0.018	2.6	44.1
4. Hydroxyapatite	10.9	256.0	0.043	6.1	22.2
5. Matrex Gel Blue A	7.2	13.8	0.522	74.6	14.7
6. Agarose Hexane CoA	4.1	1.0	4.100	585.7	8.4

Sepharose CL-6B column equilibrated with 7 mM Tris-HCl (pH 9.5), 1 mM EDTA, 0.1% Tween-20 (buffer B). The sample was eluted with a 0–0.4 M NaCl linear gradient in buffer B. Fractions containing CPT were combined and dialized against 10 mM potassium phosphate (pH 6.5), 1 mM EDTA, 0.1% Tween-20 (buffer C). The sample was applied to CM-Sepharose column equilibrated with buffer C. The void fraction was brought to pH 7 with a few drops of 5 N KOH and applied to a hydroxyapatite column equilibrated with 10 mM potassium phosphate (pH 7), 0.1% Tween-20 (buffer D). A 0.01–0.4 M linear gradient of potassium phosphate (pH 7), 0.1% Tween-20 was used for the elution. Eluted CPT was dialized against 10 mM potassium phosphate (pH 8), 0.5 mM EDTA, 0.1% Tween-20 (buffer E). The sample was applied to a Matrex Gel Blue A column equilibrated with buffer E. A 0–0.8 M linear gradient of NaCl in buffer E was used for the elution. Fractions containing CPT were pooled, dialized against 5 mM potassium phosphate, 0.5 mM EDTA, 0.1% Tween-20 (buffer F) and applied to an Agarose Hexane-CoA column equilibrted with buffer F. The sample was eluted with a 0.005–0.150 M linear gradient of potassium phosphate pH 7, 0.1% Tween-20. The native molecular mass of CPT was determined by gel filtration using a Sephacryl S-300 column calibrated with gel filtration standards (Pharmacia). To determine the subunit molecular mass, CPT purified from human liver was electrophoresed on a 10% SDS-polyacrylamide gel [11]. The gel was stained with 0.25% (w/v) Coomassie brilliant blue and destained with 10% (v/v) acetic acid/40% (v/v) methanol solution. The band obtained was then compared with standard proteins of high molecular mass (BioRad). The isoelectric point was determined by Ampholine PAG-plates pH 3.5–9.5 (Pharmacia-LKB) [12].

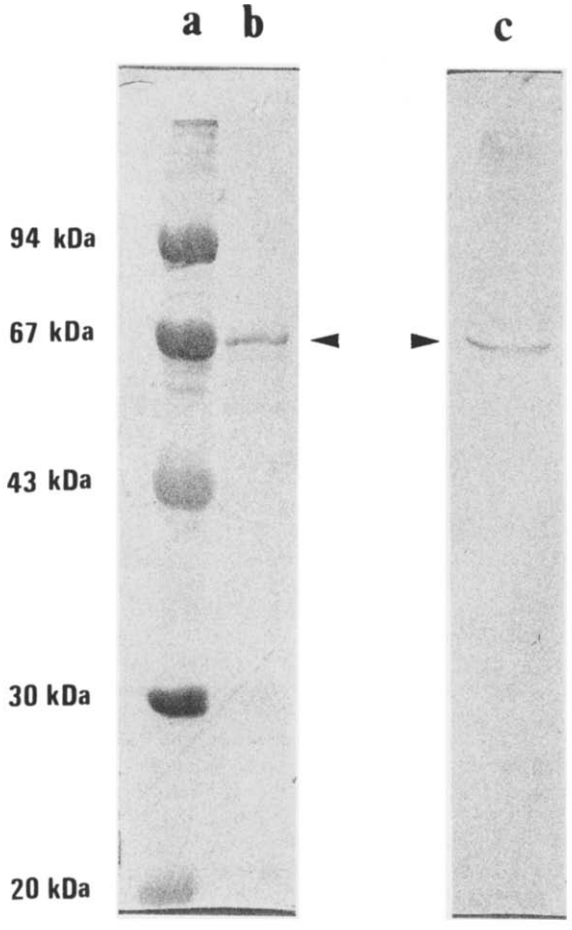

Fig. 1. Markers of molecular mass and two aliquots of purified CPT (~ 5 μg each) were loaded onto a 10% SDS-PAGE slab gel and blotted onto nitrocellulose. One part of the filter was stained with Coomassie blue (lane a, M_r markers; lane b, purified CPT). The other was matched with 100-fold diluted anti-CPT antiserum, and the immunoblot was performed as described in [17] (lane c).

2.3. *Preparation of anti-CPT antisera*

One ml of a mixture containing 0.5 mg of purified CPT and Freund adjuvant were injected in the back of white New-Zealand rabbit. The animal was boosted twice with 0.25 mg of CPT solution and semicomplete adjuvant and bled 10 days after the last booster.

2.4. *Isolation and sequencing of tryptic peptides*

Tryptic digestion and HPLC analysis of purified human CPT (132 μg) was performed as described [13]. For the determination of the N-terminal sequence we used CPT blotted onto Immobilon membranes (Amicon) [14].

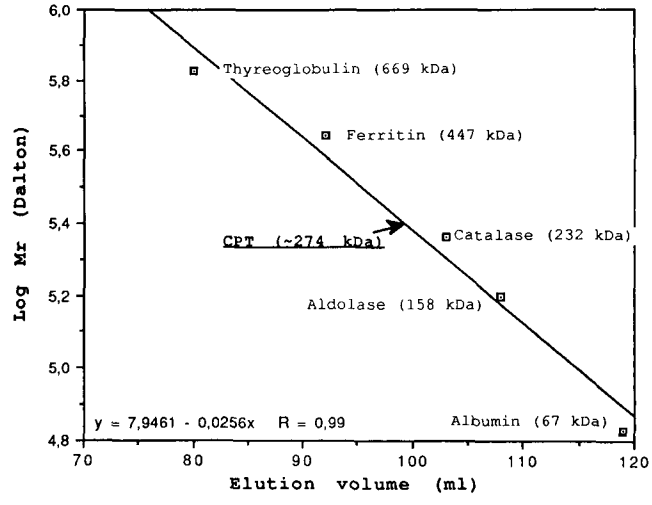

Fig. 2. Plot of the log of M_r markers (vertical axis) and elution volume from Sephacryl S-300 column (horizontal axis). The elution volume of human CPT (98 ml) is indicated by the arrow.

Table II

Kinetic profiles of CPT from human liver

Substrate	K_m (μM)	V_{max}
Acetyl-CoA	-	0.5
Hexanoyl-CoA	151.5	2.1
Octanoyl-CoA	22.9	3.4
Decanoyl-CoA	16.7	6.3
Lauroyl-CoA	11.0	7.9
Myristoyl-CoA	30.8	5.5
Palmitoyl-CoA	12.2	4.1
Stearoyl-CoA	18.0	0.7
L-carnitine with 0.1 mM octanoyl-CoA	5014	
L-carnitine with 0.1 mM lauroyl-CoA	400	
L-carnitine with 0.1 mM myristoyl-CoA	593	
L-carnitine with 0.1 mM palmitoyl-CoA	386	
L-carnitine with 0.1 mM stearoyl-CoA	348	
Palmotoyl-L (-)-carnitine	123	
Octanoyl-L (-)-carnitine	1139	

3. RESULTS AND DISCUSSION

CPT has been purified to homogeneity from beef heart and rat liver mitochondria [8,15], but never from human tissues. We purified human CPT by enriching its activity almost 600 times over that of the liver membrane-fraction extracted with Tween-20 (Table I).

The final purified preparation showed a 66 kDa band, as determined by SDS-PAGe (Fig. 1, lane 2), while rat liver CPT seems to be 1–2 kDa larger than its human counterpart [16]. Gel filtration experiments indicated that native human CPT has a molecular mass of 274 kDa, suggesting that this enzyme is a homotetramer (Fig. 2). These observations are in agreement with those obtained from CPT purified from rat liver mitochondria [8].

The pH optimum for CPT resulted to be 8.5. The isoelectric point was 6.4. The comparison of the kinetic parameters shown in Table II with those of human carnitine acetyl-transferase [17] and rat carnitine octanoyl-transferase [8], clearly indicate that the protein we purified is indeed CPT.

We checked whether purified human liver CPT was inhibited by malonyl-CoA. No inhibition was observed, in agreement with the observations by Woeltje, K.F. et al. [16] that rat liver CPT extracted by Tween-20 is not inhibited by malonyl-CoA. The lack of inhibition was observed not only with palmitoyl-Coa but also with octanoyl-CoA as a substrate, a result which is different from that obtained with CPT [18]. Whether this lack of inhibition is due to the separation of the purified enzyme from its membrane environment or to the existence of another, malonyl-CoA inhibitable CPT isoenzyme, is still controversial.

We raised polyclonal antibodies against human CPT. The antiserum obtained is effective for both immunoblotting (Fig. 1, lane c) and immunoprecipitation of human CPT and is therefore suitable for immunological studies of patients affected by CPT deficiency. Seven peptides out of 163 obtained from tryptic digestion of human CPT were sequenced, yielding 148 amino acidic residues. Furthermore, human CPT blotted onto Immobilon was also used for the determination of the N-terminal sequence, yielding 14 more residues. As a whole, these sequences account for more than 25% of the sequence of mature CPT.

Their comparison with the amino acid sequence of rat liver CPT [18] shows a high degree of homology, ranging from 78.6% to 96.2% (Table III). All the peptides sequenced were therefore derived from CPT,

Table III

Amino acid sequences of CPT from human liver*

Peptide 93:	DIWAELR (85.7%)
Peptide 115:	HTSYISGPWFDMYLSAR (94.1%)
Peptide 124a:	GNFYIFDVLDQDGNIVSPSEIQAHL (84%)
Peptide 124b:	DSVVLNFNPFMAFXP (92.9%)
Peptide 130a:	GIILPELYLDPAYGQINHNVLSTSTLSSPAVNLGGFAPVVSDGF (81.8%)
Peptide 130b:	QKLSPDAVAQLXFQMXXL (86.7%)
Peptide 132:	FVPSSLSWYGAYLVNAYPLDMSQYFR (96.2%)
N-terminal:	SAGSGPGQYXQXSIVP (78.6%)

*The homology with the corresponding sequence in rat liver CPT [14] is shown in parentheses.

strongly suggesting that the purified enzyme was essentially free of contaminants.

The definition of human CPT properties and the availability of anti-human CPT antibodies should allow a deeper understanding of the structural basis of clinical heterogeneity in CPT deficiency. Furthermore, the partial amino acid sequences obtained from purified CPT are an indispensable tool for the identification of the cDNA encoding such an enzyme.

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