Development and characterization of a polyclonal antibody against rat liver mitochondrial overt carnitine palmitoyltransferase (CPT I)

Distinction of CPT I from CPT II and of isoforms of CPT I in different tissues

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The [^3H]tetradecylglycidyl-CoA (TDG-CoA)-binding protein (M_r approx. 88 000) of purified outer membranes from rat liver mitochondria was identified by SDS/PAGE. The region in which it migrated was shown to contain another protein which stained strongly with periodic acid–Schiff reagent and could be removed from membrane extracts by incubation with Sepharose–concanavalin A. Amounts of TDG-CoA-binding protein were prepared from lectin-treated extracts using preparative SDS/PAGE and used to raise a polyclonal antibody in a sheep. The IgG fraction purified from this antiserum reacted strongly with a protein of M_r ~ 88 000 on Western blots, and much more weakly with two other proteins of M_r ~ 76 000 and M_r ~ 53 000 in extracts of rat liver mitochondrial outer membranes. The crude IgG fraction and immunopurified IgG both removed carnitine palmitoyltransferase (CPT) I activity from very pure outer membrane extracts, suggesting that the TDG-CoA-binding protein against which the antiserum was raised also expresses CPT I activity. This was confirmed by the demonstration of a strong positive correlation between CPT I activity and the amount of immunoreactive protein of M_r ~ 88 000 in mitochondria prepared from rats in different physiological states. By contrast, the antibody did not react with CPT II either in mitochondria or in purified form. Similarly, an anti-(CPT II) antibody did not cross-react with CPT I on Western blots, proving conclusively that CPT I and CPT II are immunologically distinct proteins, as well as being of different functional molecular sizes [Zammit, Corstorphine & Kelliher (1988) *Biochem. J.* **250**, 415–420]. Immunoblots of mitochondrial proteins obtained from different tissues indicated that, of the rat tissues tested, only kidney cortex mitochondria contain the same isoform of CPT I as that in liver. Heart, skeletal muscle and brown adipose tissue mitochondria contain a slightly smaller isoform which was only weakly reactive with anti-(rat liver CPT I) antibody, indicating that these tissues contain a molecularly quite distinct isoenzyme. This would explain the previous observations that CPT I in these tissues has markedly different kinetic characteristics from the isoenzyme present in liver mitochondria.

INTRODUCTION

Considerable controversy has surrounded the gradual elucidation of the relationship between the different components of the mechanism whereby mitochondria transfer long-chain acyl moieties from the cytosol into the matrix. Although it has been recognized for a considerable time [1,2] that two carnitine acyltransferases are required on either side of the permeability barrier to acyl-CoA esters presented by the inner membrane, the molecular identity or otherwise between these two transferases [overt (I) and latent (II)] has been disputed. The more amenable of the two transferases to characterization, carnitine palmitoyltransferase (CPT) II, was purified to homogeneity in several laboratories, and, more recently, its complete nucleotide sequence was described for the rat and human liver enzyme [3,4]. It has a molecular size of approx. 69 000.

Parallel progress on elucidation of the structure of CPT I has been hampered by the difficulty in solubilizing this protein from the mitochondrial outer membrane and by the hitherto unsuccessful attempts to raise specific antibodies against it (see [5]). Polyclonal antibodies raised against CPT II have been variously found to precipitate all CPT activity in solubilized mitochondria [6] and outer membranes [7], but not to react with any proteins on Western blots after SDS/PAGE of detergent-solubilized mitochondrial outer membranes enriched in CPT I activity [8].

Consequently, disparate conclusions about the similarity between the two proteins have been reached. Indirect evidence that CPT I and CPT II have different molecular sizes was obtained from observations that irreversible inhibitors of CPT I, such as tetradecylglycidyl-CoA (TDG-CoA) and etomoxir, bind to a protein of M_r ~ 90 000 [9–11] in isolated mitochondria, whereas pure CPT II has an M_r of 69 000 (see above). This evidence is indirect as it relies on the (not implausible) assumption that [^3H]TDG-CoA binds at the active site of CPT I [10]. It has also been shown [11] that [^3H]etomoxir binding capacity co-elutes with CPT activity when octyl glucoside extracts of outer membranes are chromatographed on hydroxyapatite, but these fractions contained many different proteins and no definitive conclusion could be reached about the identity of the two proteins (i.e. [^3H]TDG-CoA-binding protein and CPT I). Indeed, some authors [12] claim that the [^3H]TDG-CoA-binding protein is a regulatory subunit of CPT I which, *in vivo*, binds malonyl-CoA. The catalytic entity represented by CPT I has been repeatedly claimed to have an M_r of ~ 69 000 by several groups [7,13,14].

Direct experimental evidence that the two catalytic entities of the transferase system, i.e. CPT I and CPT II, do have different molecular sizes was finally obtained using target size analysis by radiation inactivation [15,16]. Using this technique we confirmed that CPT II has an M_r of approx. 69 000, and further showed that

Abbreviations used: CPT, carnitine palmitoyltransferase; TDG-CoA, tetradecylglycidyl-CoA; PBS, phosphate-buffered saline; COT, carnitine acyltransferase.

CPT I has an M_r between 83 000 [16] and 97 000 [15]. This size for CPT I places it within the range observed by others for the [^3H]TDG-CoA-binding protein [9–11]. These experiments constituted strong evidence that the CPT I catalytic and TDG-CoA-binding activities reside in the same protein. However, direct experimental proof of this has not been possible because of the difficulty hitherto encountered in preparing an antibody against either the TDG-CoA-binding protein or CPT I catalytic activity [5].

The aims of the present study were, therefore, two-fold: first, to develop and characterize an antibody raised against the TDG-CoA-binding protein and test whether this also recognizes CPT I, and secondly, to use the antibody to study the immunological relationship between CPT I isoforms in different rat tissues. The results provide the strongest evidence yet that CPT I and the TDG-CoA-binding protein are identical and that immunologically distinct isoforms of CPT I occur in different tissues. We also demonstrate that the anti-(CPT I) antibody does not cross-react with CPT II.

MATERIALS AND METHODS

Animals

Wistar rats were used (see [15]), and were maintained on a commercial chow diet [15]. Outer membranes for electrophoretic separation of CPT I were prepared from male rats (450–550 g) that had been starved for 48 h. Mitochondria for immunoquantification of CPT I were prepared from female rats (160–350 g) in various (patho)physiological states, as indicated. Diabetes (blood glucose > 25 mM) was induced by intraperitoneal injection of streptozotocin (80 mg/kg body wt.) 72 h before use.

Preparation of mitochondria and outer membranes

All of the liver mitochondrial preparations involved differential centrifugation followed by density gradient purification on gradients generated in 31 % Percoll (see [16,17]). After removal of the Percoll by washing, the mitochondria either were used for SDS/PAGE (for quantification of CPT I, see below) or were processed further for the purification of outer membranes. This was performed as described previously [17] and included the Sepharose-cytochrome *c* affinity purification step. Mitochondria from other tissues were prepared by differential centrifugation according to methods described previously (brain [18], heart [19], skeletal muscle [20], kidney [21], mammary gland [22], brown adipose tissue [23] and white adipose tissue [24]).

SDS/PAGE separation of proteins

Purified intact mitochondria or outer membranes were disaggregated in 8 M-urea/62.5 mM-Tris/HCl (pH 6.8)/3 % SDS, containing 5 % mercaptoethanol, by heating (70 °C for 3 min) and sonication (3 × 20 s) using a Kontes sonicator fitted with a microprobe and operating at 70 % of maximum power. Boiling of the samples at this stage was avoided, as it resulted in aggregation and loss of CPT I due to non-permeation into the separating gel. Routinely, 3 % and 7.5 % polyacrylamide gels were used for stacking and separation respectively.

Detection of [^3H]TDG-CoA-binding protein

Outer membranes (0.5–1 mg of protein) were incubated at 20 °C with 5 μM -[^3H]TDG-CoA in a medium containing 130 mM-KCl, 5 mM-Tris and 1 mM-EGTA (pH 7.4 at 20 °C). They were then separated by centrifugation (150 000 *g*, 20 min), washed with 1 % BSA in KCl buffer (150 mM-KCl/5 mM-Tris/1 mM-EGTA, pH 7.4 at 0 °C) and re-sedimented. Total membrane proteins were then sedimented using cold acetone and were

disaggregated as described above. After electrophoresis the gels were fixed, treated with En⁺Hance (Du Pont) and exposed to Kodak X-Omat AR film, according to the manufacturer's instructions.

Antibodies to CPT-I and CPT II

The band corresponding to the [^3H]TDG-CoA-binding protein on SDS/PAGE was identified from autoradiographs. Preliminary studies (see the Results section) indicated that this band contained another protein which could be removed by prior treatment of outer membrane extracts with concanavalin A. Therefore separation of polypeptides was performed routinely on extracts of outer membranes (2 mg of protein/ml) that had been solubilized in 15 mM-sodium cholate, 150 mM-NaCl, 50 mM-Tris (pH 7.4), 10 mM-dithiothreitol, 1 mM-MgCl₂, 1 mM-MnCl₂ and 1 mM-EGTA (30 min at 0 °C, with 30 s of sonication every 10 min). The extract was treated with Sepharose-concanavalin A (0.2 ml/ml of extract). After separation of the Sepharose-concanavalin A by centrifugation (500 *g*, 5 min), the proteins in the soluble phase were disaggregated and electrophoresed, as described above. With practice it was possible to identify the band corresponding to the protein without prior labelling with TDG-CoA in unfixed gels in which the protein bands were visualized after soaking in 0.25 M-KCl [25]. Routinely, approx. 1 μg of protein was obtained from 4–6 mg of outer membrane protein. To raise antiserum, the band was excised from several preparative scale gels and the protein was electroeluted into buffer containing 25 mM-Tris, 190 mM-glycine and 0.1 % SDS (pH 8.3). After conjugation of the protein to keyhole limpet haemocyanin using glutaraldehyde [26], it was used to immunize a sheep using standard techniques. A sample of pre-immune serum was obtained prior to starting the injections of the animal with antigen. CPT II was purified from rat liver mitochondria as described previously [7] and a polyclonal antibody was raised in a sheep using standard techniques.

Preparation of IgG fraction and affinity purification

Immune and pre-immune sera were treated with (NH₄)₂SO₄ to remove albumin and other non-IgG proteins. The insoluble protein was removed by centrifugation at 10 000 *g* for 30 min. The IgG in the supernatant was precipitated with (NH₄)₂SO₄ and the final pellet was suspended in phosphate (10 mM)-buffered saline, pH 7.4 (PBS), and dialysed against the same medium.

Aliquots of the final protein solution were diluted 1:100 with PBS containing 0.2 % fat-free dried milk powder. This solution was incubated either for 2–3 h at room temperature or for 16 h at 4 °C with nitrocellulose strips on to which the antigen had been electroblotted from preparative SDS/PAGE gels [27]. After washing, the antibody bound to the nitrocellulose strips was eluted with 0.2 M-glycine (pH 2.8) by incubation at room temperature for 2 h, and neutralized to pH 7.0 using NaOH. Protease inhibitors (0.1 mM-phenylmethanesulphonyl fluoride, and 1 μg each of antipain, pepstatin and leupeptin/ml) were added, and the mixture was dialysed against PBS at 4 °C for 16 h. The dialysed material was concentrated, using Centricon-100 microconcentrators, centrifuged at 2000 *g* for the required time, and stored at –20 °C.

Detection and quantification of CPT I on Western blots

The component proteins of rat liver mitochondria prepared and purified from rats in different physiological states were separated by SDS/PAGE. After blotting on to nitrocellulose (Highbond C-extra; Amersham), a standard quantity of sheep IgG was spotted on to the blot for calibration purposes. The blots were then blocked with 2 % fat-free milk protein in PBS and washed. They were incubated with affinity-purified antibody

solution (1:100) in PBS containing 0.2% fat-free dried milk for 2 h at room temperature, and washed thoroughly with PBS. The blots were then placed in 50 ml of PBS containing ^{125}I -labelled Protein G (500 000 c.p.s.) and incubated at room temperature for 2 h. Finally they were washed, dried and exposed to Kodak X-Omat AR film overnight at -80°C . The immunoreactive bands and the IgG standards were localized and excised for counting. Appropriate blanks were obtained concurrently. The linearity of the response was ascertained by electrophoresing at least two concentrations of each protein sample.

Reaction of antibody with CPT I in detergent extracts of outer membranes

Purified outer membranes were solubilized by incubation (1 h at 0°C , 3×20 s periods of sonication) in a medium containing 10 mM-octyl glucoside, 150 mM-KCl, 5 mM-Tris (pH 7.4), 1 mM-EGTA and protease inhibitors (see above). The unsolubilized membrane material was removed by centrifugation at 100 000 *g* for 30 min. Aliquots of the supernatant were incubated with increasing amounts of anti-(CPT I) IgG or PBS. The incubations were performed in the presence of 10 mM-octyl glucoside for 1 h at 20°C . A suspension (0.1 ml) of Sepharose-Protein G was added and the incubation was continued for a further 30 min, followed by centrifugation at 1000 *g* for 10 min to sediment the Sepharose. The activity of CPT I remaining in the supernatant was assayed radiochemically (see below). Parallel experiments, in which identical quantities of purified IgG fraction obtained from pre-immune serum were used, served as controls.

Assay of CPT I and CPT II activities

These were all performed radiochemically. Overt CPT (CPT I) activity was measured in the direction of palmitoylcarnitine synthesis in intact mitochondria, using palmitoyl-CoA as substrate. The assay conditions were: 150 mM-KCl, 5 mM-Tris (pH 7.4), 1 mM-EGTA, 1 mM-dithiothreitol, 5 mM-MgCl₂, 5 mM-ATP, 100 μM -palmitoyl-CoA, 0.13% fatty acid-free albumin, 1 μg of rotenone/ml and 1 μg of antimycin A/ml. Mitochondria (1–2 mg) were added to the assay medium (final volume 2.0 ml) at 37°C and incubations were continued for 2 min before addition of [^3H]carnitine (0.5 mM, 0.5 Ci/mol) to start the reaction. Assays were performed for 1–2 min and terminated by addition of 0.3 ml of 6 M-HCl. The [^3H]palmitoylcarnitine formed was quantified as described previously [28]. CPT II activity was measured in Tween-20 extracts of whole mitochondria. Known amounts of purified mitochondria (1–2 mg of protein) were treated with Tween-20 (3 μl of detergent/mg of protein) for 40 min at 0°C , with two periods (10 s) of sonication. This treatment was found to solubilize all CPT II activity but no CPT I activity by immunoblots (results not shown), as expected from previous observations [8]. The subsequent assay of CPT II activity in the supernatant was performed as described above.

Other assays

The purity of outer membrane preparations was routinely checked by assay of catalase, uricase, NADPH-cytochrome *c* reductase, monoamine oxidase and succinate dehydrogenase, as described previously [18]. No detectable contamination by peroxisomal or microsomal markers was observed, and monoamine oxidase in the outer membrane fractions was enriched approx. 200-fold with respect to whole mitochondria on a protein basis.

Materials

These were as described previously [15,17]. In addition, ^{125}I Cl was obtained from Amersham International (Amersham, Bucks., U.K.); Sepharose-Protein G and Protein G were obtained from

Sigma (Poole, Dorset, U.K.). ^{125}I -labelled Protein G was prepared as described in [29]. [^3H]TDG-CoA was synthesized as described in [30], except that [11,12(n)- ^3H]tetradecan-1-ol (produced by tritiation of tetradecan-1-ol by Amersham International) was converted into compound III of [30] by reaction with methane sulphonylchloride in pyridine. Characterization of intermediate products in the synthesis and the final material (TDG acid) was by mass spectrometry and i.r. spectroscopy as described in [30]. The CoA ester was synthesized as described by [30].

RESULTS AND DISCUSSION

Labelling of purified rat liver mitochondrial outer membranes with [^3H]TDG-CoA resulted in the detection of a single radioactive band (M_r approx. 88 000) on fluorographs of SDS gels on which the proteins were separated (Fig. 1). This size was very similar to that quoted for the [^3H]TDG-CoA binding protein of rat liver by several other workers [9–11] and to the size of the CPT I catalytic activity determined by target size analysis [15,16]. Preliminary experiments established that this region of the SDS gels in which the [^3H]TDG-CoA-labelled protein occurred also stained positive when gels were treated with periodic acid-Schiff reagent, suggesting the presence of a glycoprotein. In order to exclude the possibility that the TDG-CoA-binding protein is a glycoprotein, we pretreated the extracts, prepared from [^3H]TDG-CoA-labelled outer membranes, with Sepharose-concanavalin A. As shown in Fig. 2, this treatment removed the glycoprotein component of this band without altering the amount of [^3H]TDG-CoA associated with it. Consequently it was concluded that the 88 000- M_r band observed on Coomassie Blue-stained gels (and with which [^3H]TDG-CoA is associated) contained at least two proteins, one of which was a glycoprotein and did not bind [^3H]TDG-CoA. Routinely we used concanavalin A-treated outer membrane extracts to obtain purified 88 000- M_r protein (p88) on SDS/PAGE, prior to electroelution and its use as antigen (see the Materials and methods section).

Fig. 1. Purification of TDG-CoA-binding protein by concanavalin A-Sepharose treatment of outer membrane extracts

Equal quantities of cholate extracts of purified [^3H]TDG-CoA-labelled outer membranes were incubated in the presence or absence of concanavalin A-Sepharose (see the Materials and methods section). After separation of the lectin (on its support), the supernatants were subjected to SDS/PAGE. The gel was stained with periodic acid-Schiff reagent (b) and, after drying, was exposed to photographic film to obtain the autoradiograph shown in (a). Lanes 1 and b, samples not exposed to concanavalin A; lanes 2 and c, samples treated with concanavalin A. Lane a contains prestained phosphofructokinase (M_r 84 000).

Fig. 2. Removal of CPT I activity from extracts of purified outer membranes by treatment with antibody against the TDG-CoA-binding protein

Purified rat liver mitochondrial outer membranes (0.12 mg of protein) were solubilized with octyl glucoside (see the Materials and methods section) and incubated with increasing amounts of IgG prepared from pre-immune (○, △) or immune (●, ▲) serum. After incubation with Sepharose–Protein G and mild centrifugation, the CPT I activity remaining in the supernatant was measured. The results of two experiments are shown in which either whole IgG fraction (▲) or immunopurified IgG (●) was used. Note that much lower concentrations of immunopurified IgG were required to effect the removal of an equivalent proportion of CPT I activity from the extracts. Open symbols denote equivalent amounts of pre-immune IgG fractions. Control samples (no added IgG) were treated with Sepharose–Protein G only.

Effects of antibody on CPT I activity in outer membrane extracts

A sheep polyclonal antibody was raised against p88 and an IgG fraction was prepared and affinity-purified (see the Materials and methods section). Preliminary experiments indicated that the antibody did not inactivate CPT I catalytic activity. Consequently, we had to use a method to test for immunoreactivity which did not depend on neutralization of catalytic activity. Because of incomplete solubilization of membranes by any of the detergents tested, it was also not possible to quantify immunoprecipitation directly. Thus we used Sepharose–Protein G treatment to remove from solution the antigen–antibody complex after mild centrifugation. When incubated with octyl glucoside extracts of purified outer membranes, the IgG fraction from the immunized sheep caused the loss of CPT I activity in the supernatant after Sepharose–Protein G treatment (Fig. 2). The IgG fraction prepared from pre-immune serum had a much smaller effect. This observation indicated that the polyclonal antibody raised against p88 is an anti-(CPT I) antibody and that p88 is CPT I as well as the [^{3} H]TDG–CoA-binding protein of rat liver mitochondrial outer membranes. This is the first direct evidence that the catalytic entity of CPT I and [^{3} H]TDG–CoA binding reside in the same protein and supports our previous observations on the target size of the protein responsible for CPT I catalytic activity in rat liver mitochondria [15,16].

Fig. 3. Immunodetection of CPT I on Western blots of extracts of purified rat liver mitochondrial outer membranes

Membranes were solubilized in cholate (see the Materials and methods section) and the solubilized proteins were electrophoresed and blotted on to nitrocellulose. The blot was probed with affinity-purified IgG from antiserum raised against the TDG–CoA-binding protein (p88). M_r markers are shown in the left-hand lane and the sizes corresponding to their migration are indicated according to the supplier's recommendations.

Fig. 4. Demonstration that anti-(CPT I) and anti-(CPT II) antibodies do not recognize CPT II and CPT I respectively on immunoblots

Two SDS/PAGE gels were run using paired amounts of protein from the samples indicated. The proteins were transferred on to nitrocellulose and one blot (lanes 1–5) was probed with anti-(CPT II) whereas the other (lanes a–e) was probed with anti-(CPT I). Key to lanes: 1 and a, whole mitochondria; 2 and b, purified outer membranes; 3 and c, peroxisomes; 4 and d, purified TDG–CoA-binding protein (p88); 5 and e, purified CPT II; m, prestained markers.

Immunoreactivity on Western blots

The antibody recognized CPT I strongly on Western blots of protein from outer membranes and of whole mitochondrial extracts even at a 1:10000 dilution (Fig. 3). At these concentrations it failed to detect any protein in the region of M_r 69000, which is the size of CPT II [6–8]. However, the antibody cross-reacted faintly with at least two other proteins, of M_r ~ 76000 and ~ 53000 (see also Fig. 1). As CPT II would be expected to be present in whole mitochondrial protein, Fig. 4 illustrates that anti-(CPT I) is specific for the outer enzyme and does not cross-react with CPT II. In support of this, antibody raised against CPT II recognized the purified 69000- M_r protein (Fig. 4, lane 5) and recognized CPT II in whole mitochondria (lane 1), but failed to recognize CPT I in either whole mitochondrial or outer membranes (Fig. 4). No CPT II was detectable in our outer membrane preparations using anti-(CPT II) antibody (Fig. 4, lane 2).

Fig. 5. Relationship between the CPT I activity in purified rat liver mitochondria and ^{125}I -Protein G labelling of the immunoreactive protein p88 (TDG-CoA-binding protein)

The total proteins of liver mitochondria from normal fed ($\blacktriangle$), 48 h-starved ($\blacktriangledown$), meal-fed ($\blacklozenge$), diabetic ($\square$), lactating ($\blacksquare$), 24 h-starved/pregnant ($\bullet$) and weaned ($\circ$) female rats were separated by SDS/PAGE and the amount of p88 detected by the antibody was quantified using ^{125}I -Protein G. The blots were exposed to photographic film and the bands migrating in the position of the TDG-CoA binding protein were localized and excised for counting of the associated radioactivity. CPT I activity was measured in the same (freshly prepared) mitochondrial samples used for electrophoresis.

A faint band was detected at the same position as CPT I in peroxisomes (Fig. 4, lane c). As the carnitine acyltransferase (COT) of peroxisomes has an M_r of ~ 60000 [31], this faint reaction is thought to represent a minor contamination of the peroxisomal fraction with outer membranes. The absence of an immunoreactive band in the $60000\text{-}M_r$ region further suggests that CPT I and peroxisomal COT are immunologically distinct. By contrast, anti-(CPT II) recognized the $60000\text{-}M_r$ band of the peroxisomal enzyme more strongly (Fig. 4, lane 3), suggesting that there is greater immunological similarity between CPT II and the peroxisomal enzyme than between CPT I and either of the other two enzymes.

When immunoprecipitation of the band of $M_r 88000$ was performed using anti-(CPT I) followed by ^{125}I -Protein G (using the same mitochondrial preparations as above), a highly significant positive correlation was obtained when ^{125}I associated with p88 was plotted against CPT I activity measured in these purified mitochondria (Fig. 5). This observation again confirms that antibody raised against the TDG-CoA-binding protein is also an anti-(CPT I) antibody.

Because of the relative closeness of the $M_r \sim 76000$ band [detected by anti-(CPT I) at low dilution] and the value of $68000 M_r$ as the size of CPT II, it was essential to prove conclusively that the $M_r \sim 76000$ band was not CPT II. Therefore purified mitochondria were prepared from livers of animals in various (patho)physiological states that were known to result in a sizeable range of CPT II expression. The activity of the enzyme was measured (see the Materials and methods section) and the amount of immunoreactive protein of $M_r \sim 76000$ was measured using ^{125}I -labelled Protein G. As can be seen from Fig. 6(a), the two parameters were not positively correlated. Indeed, there was a modest degree of negative correlation, thus proving that the $M_r \sim 76000$ band was not CPT II and that anti-(CPT I)

Fig. 6. Correlation between CPT II activity in rat liver mitochondria and the amount of the minor immunoreactive protein ($M_r 76000$) detected by anti-(CPT I) antibody (a) and by anti-(CPT II) antibody (b)

The same protocol as described in the legend to Fig. 5 was used, except that CPT II activity and ^{125}I associated with either the $76000\text{-}M_r$ protein band detected by anti-CPT I (a) or that associated with the $68000\text{-}M_r$ band detected by anti-CPT II (b) were determined. Rats used: $\blacktriangle$, normal fed; $\blacktriangledown$, 48 h-starved; $\blacklozenge$, meal-fed; $\square$, diabetic; $\blacksquare$, lactating; $\bullet$, 24 h-starved/pregnant; $\circ$, weaned.

did not detect CPT II on Western blots. As expected, there was a strong correlation between CPT II activity and ^{125}I associated with the $M_r 68000$ protein detected by anti-(CPT II) (Fig. 6b).

The identity of the $M_r \sim 76000$ and $M_r \sim 53000$ proteins must remain speculative at present. It is possible that these two outer membrane proteins also utilize long-chain acyl-CoA as substrate and would thus have some commonality of epitopes with CPT I due to the acyl-CoA binding site. In this respect, long-chain acyl-CoA synthetase (EC 6.2.1.3; $M_r \sim 78000$ [32]) and glycerol phosphate acyltransferase (EC 2.3.1.15; $M_r \sim 54000$ [33]) both occur in the mitochondrial outer membrane and may, therefore, also have common outer membrane anchoring domains.

Fig. 7. Anti-(CPT I) immunoblots of mitochondrial extracts prepared from different rat tissues

Key to source of mitochondria; lane 1, heart; 2, skeletal muscle; 3, foetal liver; 4, adult liver; 5, kidney; 6, white adipose tissue; 7, brown adipose tissue; 8, mammary gland; 9, brain. Equivalent amounts of protein were loaded in each lane. The positions to which pre-stained markers migrated are indicated.

Fig. 8. Anti-(CPT II) immunoblots of mitochondrial extracts prepared from different rat tissues

A duplicate blot to that generated for the data shown in Fig. 7 was prepared and probed with anti-(CPT II). Lane 1, heart; 2, skeletal muscle; 3, foetal liver; 4, adult liver; 5, kidney; 6, white adipose tissue; 7, brown adipose tissue; 8, mammary gland; 9, brain.

Detection of CPT I in various rat tissues

Mitochondria were prepared from eight different adult rat tissues and from foetal liver. Equivalent amounts of mitochondrial protein from each tissue were loaded on to SDS gels, and the relevant blots were probed with either anti-(CPT I) or anti-(CPT II) antibodies. Optimal sensitivity was obtained by detecting the immunoreactive bands using ^{125}I -Protein G. It can be seen (Fig. 7) that only kidney cortex mitochondria contain a strongly reactive CPT I detectable by the antibody raised against liver CPT I.

The extract from heart showed a very weakly reacting band that was about 3000- M_r smaller than the CPT I in liver. This size corresponds to the smaller ^3H TDG-CoA-binding protein described previously [8]. Thus, although rat heart mitochondria express higher CPT I activity per mg of protein than do liver mitochondria [34] (see also below), the heart enzyme is very weakly detected by anti-(rat liver CPT I). This suggests that liver

and heart CPT I enzymes are largely immunologically distinct proteins. The same holds for the enzyme from skeletal muscle. This observation is highly significant. First, it has been known for some time that the rat liver enzyme has very different kinetic characteristics (with respect to malonyl-CoA inhibition [34] and K_m for carnitine [35]) from those of the enzymes in heart and skeletal muscle mitochondria. Secondly, the ^3H TDG-CoA-binding protein in these latter two tissues was shown to be smaller than that in the liver [8]. The present data demonstrate that these differences are due to the fact that the proteins that catalyse the CPT I reaction in the two sets of tissues are sufficiently different to be distinguished immunologically. This is probably also the case for CPT I in brown adipose tissue. The activity of the enzyme in mitochondria from brown adipose tissue is expected to be high, and yet the antibody reacted very weakly with protein in the 85000–90000- M_r region (Fig. 7). The presence of appreciable acylcarnitine-metabolizing capacity in these mitochondria was indicated by the large amounts of immunoreactive CPT II observed when the equivalent blots were probed with anti-(CPT II) (Fig. 8). Interestingly, brain mitochondria appear to contain the same immunological isoforms as do heart and skeletal muscle mitochondria. No conclusions could be reached about the form of CPT I in mammary gland and white adipose tissue, as the amounts of CPT I (and of CPT II) were evidently too low to be detectable.

In the more lipogenic tissues, the $M_r \sim 76000$ band was particularly prominent, suggesting that this indeed represents a protein involved in the lipogenic pathway (see above). The M_r 53000 protein was also fairly abundant in these tissues. An additional band ($M_r \sim 55000$) was detected by anti-(CPT I) only in mitochondria from mammary tissue.

Foetal rat liver mitochondria were studied because it has been suggested [36] that CPT I in these mitochondria may be of a different type from that in adult liver. However, it is evident that these mitochondria contain CPT I of an identical size and immunoreactivity as that in adult liver.

Fig. 8 also illustrates that, as expected from the data reported in [5], CPT II in all the tissues tested had the same M_r (approx. 68000). However, it was noticeable that for an equivalent quantity of mitochondrial protein, much fainter reactions with anti-(CPT II) antibody were obtained with mitochondria obtained from kidney cortex, white adipose tissue, mammary gland and brain. Whereas it is possible that for the latter three tissues this may be related to the low fatty acid-oxidizing capacity of these mitochondria, the same is not likely to hold for kidney cortex mitochondria. This raises the prospect that, although CPT II in all rat tissues may be of approximately the same M_r , there may be immunologically distinct isoforms in different tissues.

Conclusions

We have developed a polyclonal antibody which reacts with rat liver mitochondrial CPT I. As the antibody was raised initially against the TDG-CoA-binding protein of rat liver mitochondria, the data strongly suggest that CPT I is also the TDG-CoA-binding protein. We have shown that CPT I in liver is immunologically distinct from that in heart and skeletal muscle, and probably also from that in brown adipose tissue, whereas CPT I in liver is identical to that found in kidney mitochondria. CPT I was shown to be immunologically distinct from CPT II using antibodies raised against the respective proteins. The development of the antibody against CPT I should provide excellent prospects for the resolution of the question as to whether malonyl-CoA binds to the CPT I protein itself or to a regulatory subunit, as well as for the further elucidation of the structure of CPT I itself.

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