Characterization of rat liver malonyl-CoA decarboxylase and the study of its role in regulating fatty acid metabolism

Jason R. B. DYCK*, Luc G. BERTHIAUME†‡§, Panakkezhum D. THOMAS*, Paul F. KANTOR*†, Amy J. BARR*†, Rick BARR*†, Dyal SINGH‡, Teresa A. HOPKINS*†, Nicolas VOILLEY‖, Marc PRENTKI‖ and Gary D. LOPASCHUK*†[1]

*Cardiovascular Research Group and Departments of Pediatrics and Pharmacology, Faculty of Medicine, University of Alberta, Edmonton, Alberta, Canada, †Lipid Lipoprotein Research Group, Faculty of Medicine, University of Alberta, Edmonton, Alberta, Canada, ‡Departments of Anatomy and Cell Biology, Faculty of Medicine, University of Alberta, Edmonton, Alberta, Canada, §Department of Biochemistry, Faculty of Medicine, University of Alberta, Edmonton, Alberta, Canada, and ‖Molecular Nutrition Unit, Department of Nutrition, University of Montreal and the CHUM, Centre de Recherche and Institut du Cancer, Montreal QC, Canada

In the liver, malonyl-CoA is central to many cellular processes, including both fatty acid biosynthesis and oxidation. Malonyl-CoA decarboxylase (MCD) is involved in the control of cellular malonyl-CoA levels, and functions to decarboxylate malonyl-CoA to acetyl-CoA. MCD may play an essential role in regulating energy utilization in the liver by regulating malonyl-CoA levels in response to various nutritional or pathological states. The purpose of the present study was to investigate the role of liver MCD in the regulation of fatty acid oxidation in situations where lipid metabolism is altered. A single MCD enzyme of molecular mass 50.7 kDa was purified from rat liver using a sequential column chromatography procedure and the cDNA was subsequently cloned and sequenced. The liver MCD cDNA was identical to rat pancreatic β-cell MCD cDNA, and contained two potential translational start sites, producing proteins of 50.7 kDa and 54.7 kDa. Western blot analysis using polyclonal antibodies generated against rat liver MCD showed that the 50.7 kDa isoform of MCD is most abundant in heart and liver, and of relatively low abundance in skeletal muscle (despite elevated MCD transcript levels in skeletal muscle). Tissue distribution experiments demonstrated that the pancreas is the only rat tissue so far identified that contains both the 50.7 kDa and 54.7 kDa isoforms of MCD. In addition, transfection of the full-length rat liver MCD cDNA into COS cells produced two isoforms of MCD. This indicated either that both initiating methionines are functionally active, generating two proteins, or that the 54.7 kDa isoform is the only MCD protein translated and removal of the putative mitochondrial targeting pre-sequence generates a protein of approx. 50.7 kDa in size. To address this, we transiently transfected a mutated MCD expression plasmid (second ATG to GCG) into COS-7 cells and performed Western blot analysis using our anti-MCD antibody. Western blot analysis revealed that two isoforms of MCD were still present, demonstrating that the second ATG may not be responsible for translation of the 50.7 kDa isoform of MCD. These data also suggest that the smaller isoform of MCD may originate from intracellular processing. To ascertain the functional role of the 50.7 kDa isoform of rat liver MCD, we measured liver MCD activity and expression in rats subjected to conditions which are known to alter fatty acid metabolism. The activity of MCD was significantly elevated under conditions in which hepatic fatty acid oxidation is known to increase, such as streptozotocin-induced diabetes or following a 48 h fast. A 2-fold increase in expression was observed in the streptozotocin-diabetic rats compared with control rats. In addition, MCD activity was shown to be enhanced by alkaline phosphatase treatment, suggesting phosphorylation-related control of the enzyme. Taken together, our data demonstrate that rat liver expresses a 50.7 kDa form of MCD which does not originate from the second methionine of the cDNA sequence. This MCD is regulated by at least two mechanisms (only one of which is phosphorylation), and its activity and expression are increased under conditions where fatty acid oxidation increases.

Key words: acetyl-CoA, diabetes, fatty acid oxidation, mitochondria.

INTRODUCTION

Malonyl-CoA, which is produced primarily by the carboxylation of acetyl-CoA by acetyl-CoA carboxylase (ACC), is central to many cellular processes, including fatty acid biosynthesis and oxidation [1–3]. In the liver, fatty acids can be synthesized at high rates, and the production of malonyl-CoA via ACC is the rate-limiting step in this process [4]. In times of nutritional excess, malonyl-CoA is produced rapidly by ACC and then converted into fatty acids by fatty acid synthase [5]. When energy supply is scarce, however, the enzymes responsible for producing malonyl-CoA and synthesizing fatty acids are inhibited [5]. This creates a well-balanced system whereby energy is either stored or utilized depending on various nutritional events.

Although the liver is thought of mainly as a biosynthetic organ, it also oxidizes fatty acids as a source of energy [5]. Malonyl-CoA is important in this process, since it inhibits carnitine palmitoyltransferase 1, the rate-limiting enzyme involved in the mitochondrial uptake of fatty acids [1,2,6]. Following inhibition of carnitine palmitoyltransferase 1, the mitochondrial uptake of fatty acids is decreased, thereby reducing mitochondrial fatty acid oxidation. During times of nutritional deficiency or diabetes, decreases in malonyl-CoA levels result in limited synthesis of fatty acids and an up-regulation of fatty acid

Abbreviations used: MCD, malonyl-CoA decarboxylase; ACC, acetyl-CoA carboxylase; MSE, mannitol/sucrose/EGTA buffer; DTT, dithiothreitol; *Cp*, corpulent gene.

[1] To whom correspondence should be addressed: 423 Heritage Medical Research Centre, The University of Alberta, Edmonton, Alberta T6G 2S2, Canada (e-mail gary.lopaschuk@ualberta.ca).

oxidation. The question remains as to how malonyl-CoA is degraded in the liver during the times when fatty acid synthase is not active.

Kolattukudy and co-workers [7,8] have identified an enzyme in both avian and mammalian tissues which is involved in the decarboxylation of malonyl-CoA to acetyl-CoA. This enzyme, termed malonyl-CoA decarboxylase (MCD), was first described as being a mitochondrial enzyme which might protect certain mitochondrial enzymes, such as methylmalonyl-CoA mutase and pyruvate carboxylase, from inhibition by mitochondrially derived malonyl-CoA [7]. Thus it was hypothesized that, in the mitochondria, propionyl-CoA carboxylase can, at low efficiency, use acetyl-CoA as a substrate to produce malonyl-CoA [7]. This, however, would seem to account for only a small fraction of the total cellular malonyl-CoA produced. The major source of malonyl-CoA is thought to originate from cytosolic ACC. Recently it has been suggested that MCD is able to regulate cytoplasmic as well as mitochondrial levels of malonyl-CoA [1,9]. Our work has shown that an increase in or maintenance of MCD activity in conjunction with a decrease in ACC activity is probably responsible for the decrease in malonyl-CoA levels and increased fatty acid oxidation seen in both the postnatal heart and the reperfused ischaemic heart [9]. Similarly, Alam and Saggerson [1] have provided indirect evidence for a cytosolic MCD activity that can alter cytosolic malonyl-CoA levels in rat skeletal muscle. These studies therefore implicate MCD as a regulator of fatty acid oxidation. Whether the observed decreases in cytosolic malonyl-CoA levels in the postnatal heart and the reperfused ischaemic heart are due to the activity of a cytoplasmic form of MCD has yet to be established.

Recently we have cloned and expressed the pancreatic β-cell form of rat MCD, which is 68% identical to MCD from the goose uropygial gland, and predicts a protein of 54.7 kDa [10,11]. Whether the liver MCD cDNA is similar to the pancreatic β-cell cDNA is not known. We do know that overall MCD activity is much higher in liver than in islet cells [10]. We have also shown that the activity of MCD is highest in tissues that oxidize fatty acids (i.e. heart and liver) and lowest in tissues that use mainly glucose as an energy source (i.e. brain) [10]. This supports the concept that MCD is involved in regulating rates of fatty acid oxidation within the cell by altering cytoplasmic malonyl-CoA levels. The cDNA for human MCD has also been cloned, and peroxisomal and cytoplasmic forms have been identified [12,13]. Although the liver has the ability to utilize excess cytoplasmic malonyl-CoA via increased fatty acid synthesis, it is unclear why this organ has a high MCD activity. Understanding the role of hepatic MCD in the regulation of fatty acid oxidation becomes essential, especially in pathophysiological states such as diabetes, where lipid metabolism is altered. We therefore purified and characterized liver MCD, and provide evidence that this enzyme has an important role in the regulation of fatty acid metabolism.

MATERIALS AND METHODS

Animals

Streptozotocin-diabetic rats

Male Sprague–Dawley rats (230–250 g) were anaesthetized with Enflurane, and then were given a single tail-vein injection of 55 mg/kg streptozotocin (Sigma Chemical Co., St. Louis, MO, U.S.A.) dissolved in 50 mM citrate, pH 4.5. Control rats were injected with vehicle alone. Animals were allowed to recover and were given access to food and water *ad libitum* for a 6-week period. Only animals demonstrating urine glucose of $> 2\%$ within 48 h of streptozotocin injection were used in the diabetic group.

Obese insulin-resistant rats

JCR:LA rats are a genetic model of insulin resistance which develops as a result of incorporating the corpulent (*Cp*) gene [14]. Homozygous (*Cp*/*Cp*) males were used as the diabetic group, while heterozygotes were used as the control group.

Fasted rats

Male Sprague–Dawley rats (230–250 g) were given access to food *ad libitum*, until the rats were either anesthetized with sodium pentobarbitol and the liver quickly excised, or subjected to a 48 h fasting period. After this time fasted rats were either anaesthetized with sodium pentobarbitol and the liver quickly excised, or subjected to a 72 h refeeding before liver extraction.

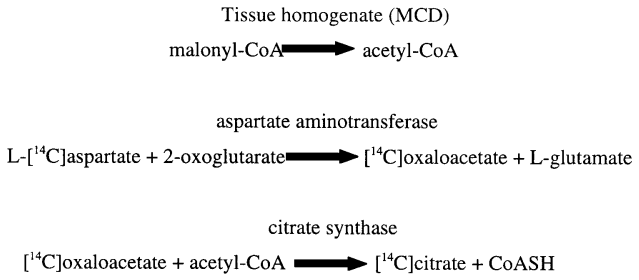
Preparation of rat liver high-density fractions

Male Sprague–Dawley rats (300–350 g) were anaesthetized with sodium pentobarbitol, and the livers were quickly excised. A total of 50 rat livers were minced into 1 mm cubes and rinsed with ice-cold MSE buffer (225 mM mannitol, 75 mM sucrose and 1 mM EGTA, pH 7.5). A ratio of 2:1 (v/w) of MSE buffer to tissue was used per liver, and tissue was homogenized using five strokes of a glass homogenizer with a Teflon pestle. The homogenate was then diluted with MSE to 10 ml/g of wet tissue and centrifuged at 480 ***g*** for 5 min. The supernatant was filtered through two layers of cheese cloth, and then centrifuged at 10000 ***g*** for 30 min. The pellet was then resuspended in 10 mM sodium phosphate buffer, pH 7.6, containing 0.5 mM dithioerythritol (5 ml/g of wet pellet). Triton X-100 (0.1%) was added to the suspension and then the slurry was stirred at 4 °C for 16 h to lyse the organelles present in the high-density fraction. The solution was then centrifuged at 24000 ***g*** for 10 min, and the supernatant saturated to 40% with $(NH_4)_2SO_4$ and stirred for 1 h on ice. The precipitated proteins were removed by centrifugation at 24000 ***g*** for 10 min. The supernatant was then saturated to 55% with $(NH_4)_2SO_4$, stirred for 1 h on ice, and centrifuged at 24000 ***g*** for 10 min. The pellet, enriched in MCD activity, was resuspended in a buffer comprising 0.1 M potassium phosphate (pH 7.4), 1 mM dithiothreitol (DTT) and 1 M $(NH_4)_2SO_4$ in preparation for the first column.

MCD assay

A fluorimetric assay which monitors the formation of acetyl-CoA from malonyl-CoA in a coupled assay using citrate synthase and malate dehydrogenase was used to measure MCD activity. Reaction mixtures of 1.4 ml contained 0.1 M Tris/HCl, pH 8.0, 1 mM dithioerythritol, 10 mM malic acid, 0.17 mM NAD^+, 0.136 mM malonyl-CoA, 11 units of malate dehydrogenase and 0.44 unit of citrate synthase. The reaction was initiated by the addition of various amounts of MCD, depending on enzyme activity (10–50 μl). The reaction measured the formation of NADH from NAD^+ over a 4 min time period using a Shimazdu RF-5000 spectrofluorimeter. The formation of NADH was measured at an excitation wavelength of 340 nm and an emission wavelength of 460 nm [15].

A radiometric MCD assay was also used for measurement of MCD activity in whole-tissue samples, prepared as described [9]. Acetyl-CoA derived from MCD was incubated in the presence of [^{14}C]oxaloacetate and citrate synthase (0.73 μ-unit/μl) to form citrate. The [^{14}C]oxaloacetate was initially produced by a 20 min transamination reaction performed at room temperature utilizing



Scheme 1 Basis of MCD assay

See the text for details.

L-[^{14}C-(U)]aspartate (2.5 μCi/ml) and 2-oxoglutarate (2 mM). To initiate the MCD assay, preparations were incubated in a 210 μl reaction mixture (0.1 M Tris, pH 8, 1 mM DTT and 1 mM malonyl-CoA) for 10 min at 37 °C, in the presence or absence of NaF (50 mM) and tetrasodium pyrophosphate (5 mM). The reaction was stopped by the addition of 40 μl of perchloric acid (0.5 mM), neutralized with 10 μl of 2.2 M $KHCO_3$ (pH 10) and centrifuged at 10000 ***g*** for 5 min to remove precipitated proteins. Incubation of the liver sample with malonyl-CoA allowed for the conversion of malonyl-CoA into acetyl-CoA, which was then combined with [^{14}C]oxaloacetate (0.17 μCi/ml) to produce [^{14}C]citrate. All reactions were carried out in the presence of *N*-ethylmaleimide, which removes excess CoA remaining in the latter stages of the reaction, so that the citrate present cannot generate non-MCD-derived acetyl-CoA. Unreacted [^{14}C]oxaloacetate was removed from the reaction mixture by the addition of sodium glutamate (6.8 mM) and aspartate aminotransferase (0.533 μ-unit/μl), followed by a 20 min incubation at room temperature. This allowed for transamination of unreacted [^{14}C]oxaloacetate back to [^{14}C]aspartate. The resulting solution was then stirred in a 1:2 (w/v) suspension of Dowex 50W-8X (100–200 mesh) and centrifuged at 400 ***g*** for 10 min. The pelleted Dowex fraction removed [^{14}C]aspartate, while the supernatant contained [^{14}C]citrate. The supernatant fraction was then counted for ^{14}C present in the form of [^{14}C]citrate. The amount of acetyl-CoA produced by MCD was then quantified by comparison with acetyl-CoA standard curves that had been obtained under identical assay conditions. A standard acetyl-CoA concentration curve was run with each experiment. These curves were always found to be linear ($r = 0.99$) (results not shown). A schematic diagram of the assay is shown in Scheme 1.

Except when treated with alkaline phosphatase, all MCD measurements were performed in the presence of phosphatase inhibitors, to avoid alterations in the phosphorylation state of MCD during the assay. In the absence of phosphatase inhibitors a modest increase in adult liver MCD activity was observed, which was significantly elevated upon addition of alkaline phosphatase (shown below).

Purification of rat liver MCD

Chromatography on butyl-Sepharose 650M

The MCD solution adjusted to 1 M $(NH_4)_2SO_4$ was loaded on to a 100 ml column of butyl-Sepharose 650 M (2.6 cm × 40 cm) previously equilibrated with a 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM DTT and 1 M $(NH_4)_2SO_4$. The column was washed with 3 column volumes of the above buffer and proteins were eluted with a gradient of 1–0 M $(NH_4)_2SO_4$ in a total volume of 150 ml, with 9 ml fractions being collected. The fractions containing MCD activity were pooled and brought to 100 ml in a buffer containing 0.1 M potassium phosphate (pH 7.4), 1 mM DTT and 1 M $(NH_4)_2SO_4$.

Chromatography on phenyl-Sepharose HP

The enzyme solution was loaded on to a 25 ml phenyl-Sepharose HP column (2.6 cm × 20 cm) previously equilibrated with a 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM DTT and 1 M $(NH_4)_2SO_4$. The column was washed with 3 column volumes of the above buffer and proteins were eluted with a gradient of 1–0 M $(NH_4)_2SO_4$ in a total volume of 175 ml, with 9 ml fractions being collected. The fractions containing MCD activity were pooled and concentrated by ultrafiltration. The resulting 1 ml solution was brought to 50 ml in a 20 mM Bis-Tris (pH 7.0) buffer containing 1 mM DTT.

Chromatography on Q-Sepharose HP

The enzyme solution was applied to a 10 ml Q-Sepharose HP column (1.5 cm × 20 cm) previously equilibrated with a 20 mM Bis-Tris (pH 7) buffer containing 1 mM DTT. The column was washed with 3 column volumes of the same buffer and bound proteins were eluted with a gradient of 0–0.3 M NaCl in a total volume of 80 ml. Fractions of 4 ml were collected, and the fractions containing MCD activity were pooled and concentrated by ultrafiltration. The concentrated pool was resuspended in 50 ml of a 50 mM Mes (pH 5.7) buffer containing 1 mM DTT.

Chromatography on SP-Sepharose HP

The enzyme solution was applied to a 5 ml SP-Sepharose HP column (1.5 cm × 20 cm) previously equilibrated with a 50 mM Mes (pH 5.6) buffer containing 1 mM DTT. The column was washed with 3 column volumes of the same buffer and bound proteins were eluted with a linear gradient of 0–0.4 M NaCl in a total volume of 150 ml. Fractions of 4 ml were collected, and the fractions containing MCD activity were pooled, neutralized to pH 7.0 and concentrated by ultrafiltration. The concentrated pool was resuspended in 5 ml of 50 mM malate (pH 5.6) buffer containing 1 mM DTT.

Malonyl-CoA affinity elution from SP-Sepharose HP

The enzyme solution was re-applied to a 0.5 ml SP-Sepharose HP column (0.5 cm × 10 cm) previously equilibrated as above. The column was washed with 50 mM malate (pH 5.6) buffer containing 1 mM DTT to remove unbound proteins, and then eluted with a 50 mM malate (pH 5.6) buffer containing 1 mM DTT and 10 μM malonyl-CoA. The eluted fractions were neutralized to pH 7.0. The affinity elution protocol allowed for highly specific elution of MCD.

Protein sequencing

The affinity-eluted protein fraction from the second SP-Sepharose HP column was resolved on an SDS/9 %-polyacrylamide gel and stained for visualization using a Coomassie Blue solution. The major protein of approx. 50.7 kDa was thought to be MCD, and was excised from the gel and subjected to an EndoLys C digest. The protein digest was subjected to HPLC and the appropriate peptides underwent amino acid N-terminal sequencing. The amino acid sequences of two internal peptides were obtained. Protein digestion and amino acid N-terminal sequencing were

performed at the Eastern Quebec Peptide Sequencing Facility (Quebec, Canada).

Production of antibody against rat liver MCD

The protein that was affinity eluted from the SP-Sepharose column was subjected to SDS/PAGE and stained with Coomassie Blue, and the 50.7 kDa band was cut from the gel. The protein (3 μg) was eluted from the gel fragment and injected into rabbits. The rabbits were injected at 2-week intervals for 2 months before the serum was used.

Cloning of rat liver MCD

Oligonucleotides based on the cloned sequence of the rat β-cell MCD [10] were designed to amplify a 900 bp product of MCD from rat liver cDNA using PCR. The forward primer, PMCDF1 (5′ TGGTCGACGGCTTCCTGAACCTG), was used with the reverse primer, PMCDR (5′ TCCCTAGAGTTTGCTGTTGCTCTG), in a PCR reaction. This 900 bp fragment was used as a probe for screening a SuperScript rat liver cDNA library (Gibco Life Technologies). The full-length cDNA clone was sequenced in both directions using internal primers and compared with the rat islet MCD cDNA sequence. Site-directed mutagenesis was performed using the Quickchange Site-Directed Mutagenesis kit (Stratagene) as described by the manufacturer's protocol.

COS-7 cell transfection

COS-7 cells were cultured in Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal bovine serum for 24 h at a density of 2×10^5 cells per well. Rat liver MCD was subcloned into the *Eco*RI restriction enzyme site of the multiple cloning site of pcDNA3.1 (Invitrogen). Cells were then transfected with pcDNA3.1 and rat liver MCD using Superfect (Qiagen) and left for 48 h. After transfection, cells were scraped into PBS, a protein assay was performed, and the cell extracts were then boiled in SDS/PAGE sample buffer.

Western blot analysis

Samples were subjected to SDS/PAGE and transferred to nitrocellulose as described previously [9]. Membranes were blocked in 10 % (w/v) skimmed milk powder/1 × PBS and then immunoblotted with anti-MCD antibodies (1:1000 dilution) in 1 % (w/v) skimmed milk powder/1 × PBS. After extensive washing, the membranes were incubated with a peroxidase-conjugated goat anti-rabbit secondary antibody in 1 % (w/v) skimmed milk powder/1 × PBS. After washing, the antibodies were visualized using the Amersham Pharmacia Enhanced Chemiluminescence Western blotting and Detection System.

Statistical analysis

The unpaired Student's *t*-test was used for the determination of statistical differences between two group means. For groups of three, ANOVA followed by the Neuman–Keuls test was used. A value of $P < 0.05$ was considered significant. All data are presented as means ± S.E.M.

RESULTS

Purification of rat liver MCD

A purification scheme for rat liver MCD was developed (see the Materials and methods section) which is substantially different from that originally described by Kolattukudy et al. [8]. This newly designed scheme allowed MCD to be purified more than 1200-fold from the initial 55 % ammonium sulphate pellet (Table 1). Due to the inability of our fluorimetric assay to detect MCD activity from whole-cell extracts, the purification profile only represents the increase in purity beginning from the high-density fraction. For this reason, MCD was actually purified to a much greater extent than we have indicated in Table 1. When pooled fractions from the columns were subjected to SDS/PAGE analysis and stained using a sensitive Coomassie Blue dye, we detected two proteins with molecular masses of 50.7 and 65 kDa (Figure 1). Previous work on mammalian MCD has suggested that the 50.7 kDa protein that we observed was MCD [9,10]. Immunoblotting experiments revealed that the second protein (which co-purifies with MCD) was catalase (results not shown).

Table 1 FPLC protocol for purification of MCD

ΔF, change in fluorescence.

Fraction	Total protein (mg)	Specific activity ($\Delta F \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	Purification (fold)
55 % $(NH_4)_2SO_4$ pellet	1459	646	1
Butyl-Sepharose 650M	544	158	2
Phenyl-Sepharose HP	288	510	8
Q-Sepharose HP	35.6	9100	29
SP-Sepharose HP	1.78	17503	271
Affinity elution	0.02	81921	1269

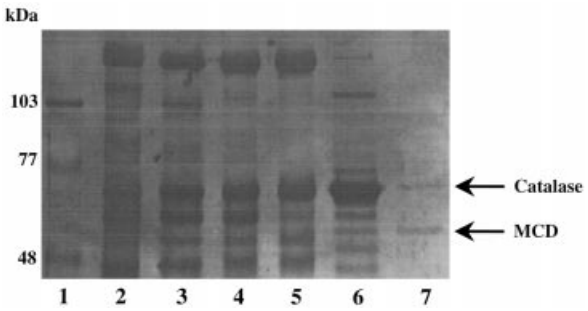


Figure 1 Coomassie Blue-stained gel of the active fractions of the MCD purification

SDS/PAGE was performed on 1 μg (except lane 7) of the active pooled fractions from each column. Molecular mass markers (lane 1) are indicated to the left (kDa). Pooled fractions from the 40–55 % $(NH_4)_2SO_4$ pellet (lane 2), butyl-Sepharose 650M column (lane 3), phenyl-Sepharose HP column (lane 4), Q-Sepharose HP column (lane 5) and SP-Sepharose HP column (lane 6), and the affinity-eluted fraction from the SP-Sepharose HP column (lane 7), are shown. The proteins corresponding to MCD and catalase are indicated.

Cloning of rat liver MCD cDNA

Oligonucleotides based on the cloned sequence of rat islet MCD cDNA [10] were designed to amplify a 900 bp product of MCD from rat liver cDNA using PCR. This 900 bp fragment was used as a probe in screening a rat liver cDNA library. A full-length (2.2 kb) clone was obtained, sequenced in both directions and compared with the rat islet MCD sequence. Sequence comparison between liver and islet forms of MCD revealed that the two forms are identical (results not shown). The two potential start sites for translation, which may produce proteins of

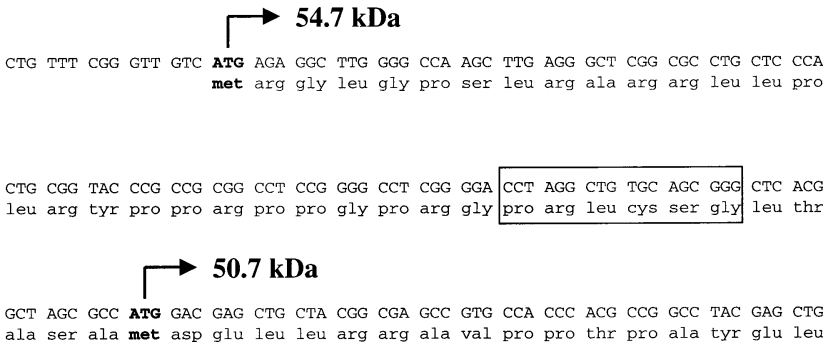


Figure 2 cDNA sequence of the N-terminus of rat liver MCD

The two initiating methionines are indicated (**bold**), along with the corresponding deduced molecular masses of the translated MCD isoforms. The boxed region indicates the putative cleavage-site motif.

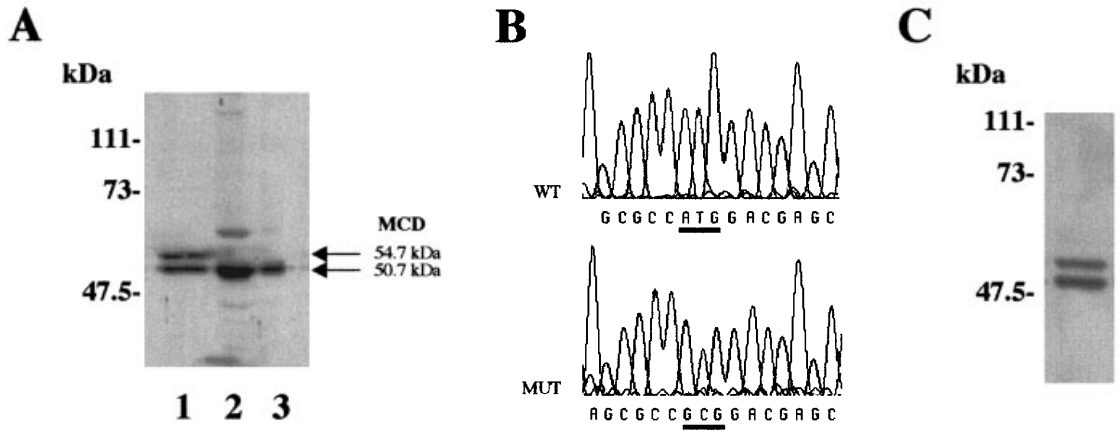


Figure 3 Western blot analysis of cell extracts from Cos-7 cells expressing wild-type and mutated rat liver MCD, rat liver homogenate and purified rat liver MCD

(**A**) Immunoblot analysis of extract from transfected COS-7 cells (lane 1), rat liver homogenate (lane 2) and purified liver MCD (lane 3) using anti-MCD antibody, revealing the molecular masses of the MCD isoforms. Transient transfection of COS-7 cells produced two MCD proteins of 50.7 kDa and 54.7 kDa, while whole-liver homogenates and purified liver MCD contained only the 50.7 kDa isoform of MCD (arrows). (**B**) Sequencing chromatograph demonstrating both wild-type and mutated forms of MCD. The mutation ATG to GCG is indicated. (**C**) Immunoblot analysis of extract of COS-7 cells transfected with mutated MCD using anti-MCD antibody, revealing the presence of two MCD proteins.

50.7 kDa and 54.7 kDa, are indicated in Figure 2. A putative cleavage-site motif (PRLCSG) [10] was also identified (Figure 2) which would theoretically produce a protein of approx. 50.7 kDa via cleavage of a 54.7 kDa translated protein.

Characterization of rat liver MCD

The fractions from the affinity elution of the SP-Sepharose column which demonstrated the highest MCD specific activity were pooled and divided into aliquots. The first aliquot was subjected to SDS/PAGE. The 50.7 kDa band was cut from the gel, digested with EndoLys C and subjected to HPLC, and the appropriate peptides underwent amino acid N-terminal sequencing. The amino acid sequences of two internal peptides were obtained and compared with the cDNA sequence obtained from the rat liver cDNA clone for confirmation. The peptide sequences obtained corresponded exactly to amino acids 336–346 and amino acids 376–385 of the amino acid sequence deduced from the cloned rat liver MCD cDNA (results not shown). The second aliquot from the affinity elution of the SP-Sepharose column was subjected to SDS/PAGE and stained with Coomassie Blue, and the 50.7 kDa band was cut from the gel. The protein was eluted from the gel fragment and injected into rabbits for the generation of a polyclonal antibody against MCD. The antibody was used in subsequent Western blot analysis (Figures 3 and 4).

Cell extracts from COS-7 cells expressing rat liver MCD were subjected to SDS/PAGE alongside rat liver homogenates and purified rat liver MCD. After transfer to nitrocellulose and subsequent immunoblotting with anti-MCD antibody, the molecular masses of the MCD proteins were determined (Figure 3A). Transient transfection of COS-7 cells produced two MCD proteins of 50.7 kDa and 54.7 kDa (Figure 3A, lane 1). In contrast, whole-liver homogenates and purified liver MCD contained only one isoform (50.7 kDa) of MCD (Figure 3A, lanes 2 and 3). Using site-directed mutagenesis, the second putative initiating ATG of MCD was mutated to GCG (Figure 3B). Transient transfection of COS-7 cells with the plasmid containing this mutated form of MCD also generated two forms of MCD (Figure 3C).

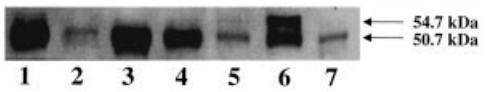


Figure 4 Tissue distribution of MCD

The protein levels of MCD were measured in a variety of rat tissues using anti-MCD antibody. Samples of 150 μg of heart (lane 1), skeletal muscle (lane 2), liver (lane 3), kidney (lane 4), lung (lane 5), pancreas (lane 6) and brain (lane 7) were boiled in a 2% SDS gel loading buffer and subjected to Western analysis. The two MCD proteins of 50.7 kDa and 54.7 kDa are indicated (arrows).

Table 2 Serum levels of glucose, fatty acids and insulin in 6-week-old streptozotocin-diabetic, JCR:LA-corpulent, control and feeding study rats

Values are means ± S.E.M. for 6–12 animals. Significance of differences: *, significantly different from appropriate control; †, significantly different from fasted ($P < 0.05$).

Rats	Glucose (mM)	Fatty acids (mM)	Insulin (m-units/l)
Diabetic control	5.74 ± 0.02	0.51 ± 0.01	87.1 ± 7.0
Diabetic	11.98 ± 0.02*	1.50 ± 0.02*	5.0 ± 0.2*
JCR lean	4.95 ± 0.26	0.39 ± 0.02	107.2 ± 17.4
JCR corpulent	5.15 ± 0.37	0.66 ± 0.05*	450.5 ± 75.1*
Feeding control	5.68 ± 0.27	0.53 ± 0.06	75.7 ± 23.5
Fasted (48 h)	3.04 ± 0.31*	1.78 ± 0.16*	8.3 ± 1.2*
Refed (72 h)	6.76 ± 0.80†	0.27 ± 0.50*†	1291 ± 16.6*†

Using Western blot analysis, the distribution of MCD in a variety of rat tissues was also determined (Figure 4). All tissues tested expressed relatively high levels of MCD protein. Oxidative tissues such as liver and heart expressed the highest levels of MCD protein, supporting the concept that MCD may play a role in controlling fatty acid oxidation [9]. The only tissue that contained two immunoreactive bands was the pancreas. This suggests that the pancreas contains two isoforms of MCD. To ensure that an isoform of MCD was not selectively removed during extraction from tissues, these experiments used detergent-solubilized whole-cell homogenates to measure total MCD levels. This prevented a potential loss of MCD protein based on cellular localization.

Phosphorylation control of MCD

Our earlier work indicated that cardiac MCD is under phosphorylation control [9]. Using our purified liver MCD and a wide variety of kinases and phosphatases, we were only able to identify alkaline phosphatase as an enzyme modifying MCD activity (results not shown). Similar experiments were performed in which we dephosphorylated and activated MCD using alkaline phosphatase and then tried to inactivate the enzyme using various kinases. All of these experiments proved to be unsuccessful (results not shown). The kinases or phosphatases tested were casein kinase II, cAMP-dependent protein kinase, protein kinase C, 5′-AMP-activated protein kinase, protein phosphatase 2A and protein phosphatase 2C.

Role of MCD in controlling fatty acid metabolism

MCD expression and activity were measured in three animal models in which alterations in fatty acid metabolism have been noted previously. Two of these are models of diabetes, in which

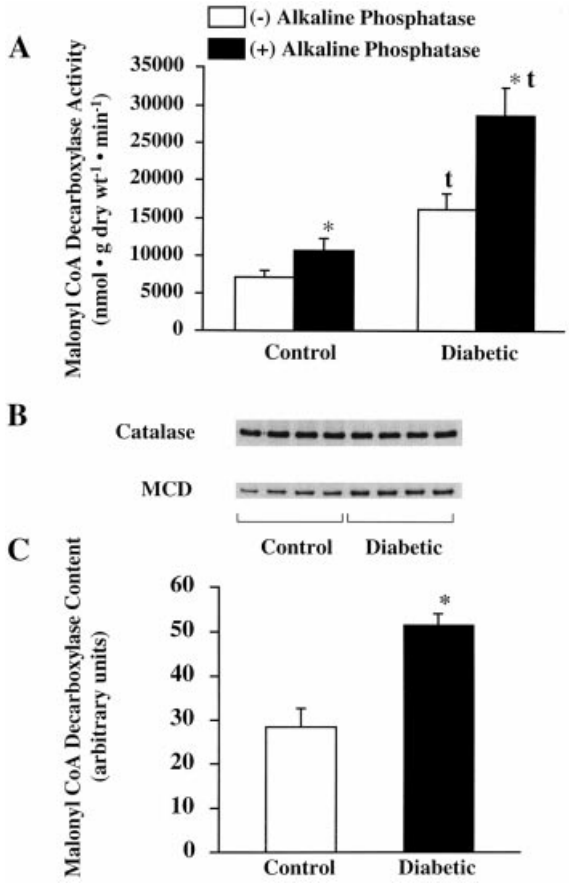


Figure 5 Rat liver MCD activity and protein levels in 6-week-old control and streptozotocin-diabetic rats

Measurement of MCD activity in the presence and absence of alkaline phosphatase treatment (**A**) and quantification of MCD protein levels (**B**) were performed using livers from 6-week-old control rats and 6-week-old streptozotocin-treated rats. The Western blots (**B**) underwent densitometry analysis, and the levels of MCD protein were quantified (**C**). Values are means ± S.E.M. of at least six livers in each group. Significance of differences: *, significantly different from non-alkaline-phosphatase-treated; t, significantly different from appropriate control.

hepatic fatty acid biosynthesis is either inhibited (6-week-old streptozotocin-diabetic rats [16]) or elevated (JCR:LA-corpulent insulin-resistant rats [17]). A group of rats was also fasted for 48 h, which both increases fatty acid oxidation and decreases fatty acid biosynthesis [18–20]. Table 2 shows the changes seen in plasma glucose and fatty acid levels in the various rat models. Serum levels of fatty acids rose 3-fold in the 6-week-old streptozotocin-diabetic rats and 1.75-fold in the JCR:LA-corpulent (*Cp/Cp*) rats as compared with their lean control groups. Similarly, in the 6-week-old streptozotocin-diabetic rats, serum glucose levels were increased 2.2-fold above those in controls. In contrast with this, the levels of glucose in the serum were unaltered in the *Cp/Cp* rats as compared with the lean controls. These changes in glucose and fatty acid levels in the serum are consistent with the pathophysiology of these animals [14,21,22].

MCD activity was increased approx. 2-fold in the 6-week-old streptozotocin-diabetic animals as compared with control rats (Figure 5A), while MCD activity was unaltered in the *Cp/Cp* rats (Figure 6A). To determine if changes in activity were due to specific post-translational modifications of MCD, such as dephosphorylation, and/or to an increase in the relative abundance

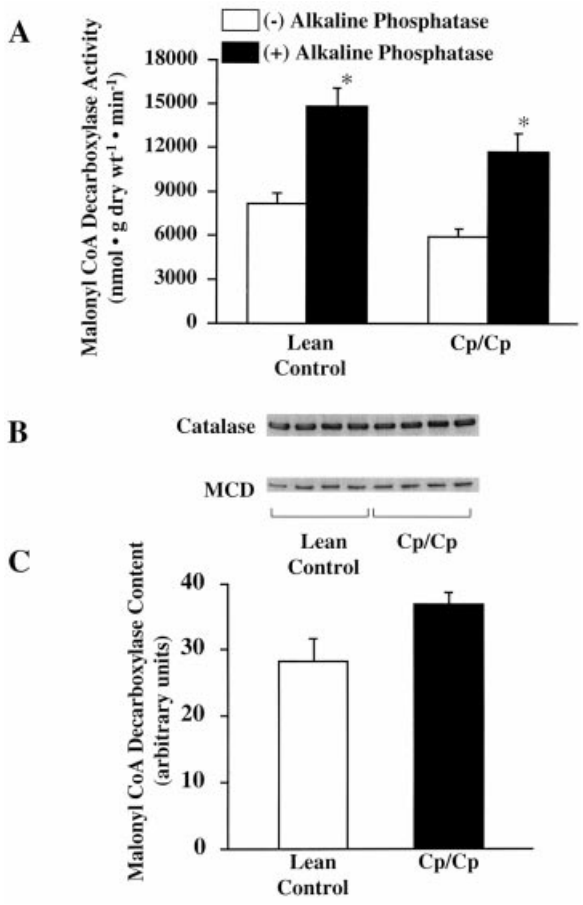


Figure 6 Rat liver MCD activity and protein levels in control and JCR:LA-corpulent rats

Measurement of MCD activity in the presence and absence of alkaline phosphatase treatment (**A**) and quantification of MCD protein levels (**B**) were performed using livers from JCR:LA-corpulent lean controls and *Cp/Cp* rats. The Western blots (**B**) underwent densitometry analysis and the levels of MCD protein were quantified (**C**). Values are means ± S.E.M. of at least six livers in each group. Significance of differences: *, significantly different from non-alkaline-phosphatase-treated.

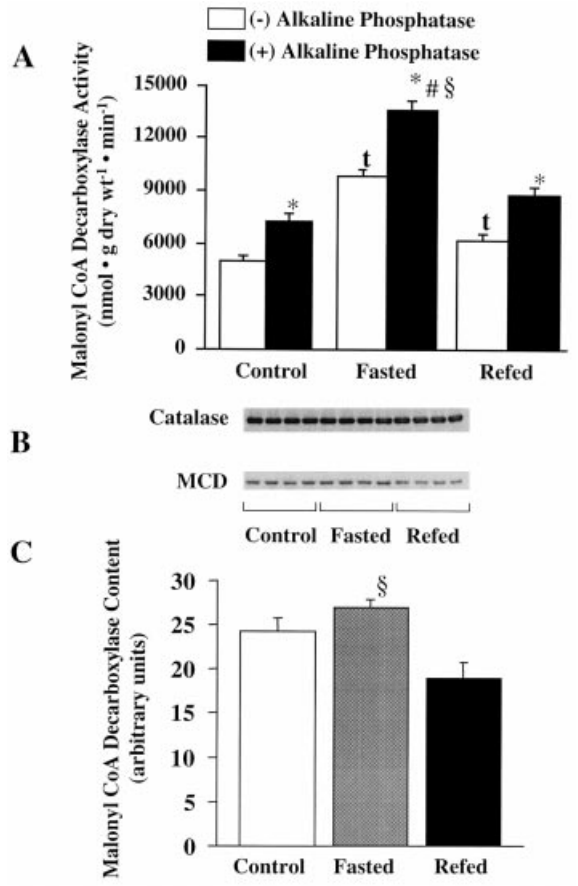


Figure 7 Rat liver MCD activity and protein levels in control, fasted and refed rats

Measurement of MCD activity in the presence and absence of alkaline phosphatase treatment (**A**) and quantification of MCD protein levels (**B**) were performed using livers from control, fasted and refed rats. The Western blots (**B**) underwent densitometry analysis and the levels of MCD protein were quantified (**C**). Values are means ± S.E.M. of at least six livers in each group. Significance of differences: *, significantly different from non-alkaline-phosphatase-treated values; t, significantly different appropriate control values; #, significantly different from control alkaline phosphatase-treated values; §, significantly different from refed alkaline phosphatase-treated values.

of the enzyme, Western blot analysis was performed using our anti-MCD antibody. The level of MCD protein was increased approx. 2-fold in the livers of 6-week-old streptozotocin-diabetic rats as compared with that in control livers (Figures 5B and 5C), while no changes were observed in the levels of MCD in the livers of the *Cp/Cp* rats (Figures 6B and 6C).

Post-translational modifications of MCD (e.g. phosphorylation control) and increased MCD protein levels seem to be involved in regulating MCD activity in the livers of diabetic rats. When liver MCD from both 6-week-old streptozotocin-diabetic rats and *Cp/Cp* rats was treated with alkaline phosphatase, we demonstrated an increased activity of the enzyme for all groups. Alkaline phosphatase treatment of liver MCD from lean control, *Cp/Cp* and control diabetic rats appeared to consistently result in MCD enzyme activity of approx. 11000–15000 nmol·min^{-1}·g dry wt^{-1} (Figures 5 and 6), indicating that alkaline phosphatase treatment is sufficient to produce a maximally active enzyme in adult rats. Only the streptozotocin-diabetic animals had higher MCD activity, which was probably due to the observed 2-fold increase in MCD protein expression.

Another well established protocol for altering fatty acid biosynthesis and oxidation in the liver is to starve and then refeed rats [18–20]. Table 2 demonstrates the significant decrease in serum glucose and increase in serum fatty acids caused by fasting. Similarly, MCD activity was increased during the 48 h fasting period (Figure 7A). When rats that had been deprived of food for 48 h were refed for 72 h, there was a decrease in MCD activity (Figure 7A). The levels of MCD activity began to approach those of control rats that had not undergone the fasting/refeeding protocol (Figure 7A). Although MCD activity varied during these various nutritional states, the level of MCD protein was not significantly altered (Figures 7B and 7C). This suggests that the activity of MCD is under post-translational regulation.

DISCUSSION

The purification of liver MCD has proved to be extremely useful in the characterization of the protein, the cloning of the cDNA and the generation of antibodies. An important observation made during the purification protocol was that MCD and catalase

appear to co-purify (Figure 1). Although the activity of MCD was independent of the amount of catalase present in the fraction, we cannot be certain of the role of catalase in regulating MCD activity. The fact that catalase is an intraperoxisomal protein which is independently targeted to the peroxisomes [23] is quite intriguing. MCD has a peroxisomal targeting sequence, and a portion of the total cellular MCD may locate to the peroxisomes, as shown by Sacksteder et al. [12]. An interaction between catalase and MCD would most probably lead to MCD being localized to peroxisomes exclusively. Since a cytosolic form of MCD has been demonstrated [12], we suggest that the co-purification of MCD and catalase may be due to similarities in the proteins, and not necessarily to any interaction between them. The role that catalase might play in MCD activity and targeting, however, is unknown.

The cDNA sequence for rat liver and β-cell MCD shows that two methionines are present within the first 120 bp of the coding region, with each having a fairly well conserved Kozak consensus sequence (Figure 2). This is similar to the reported goose MCD sequence, where the two separate start codons are able to code for two proteins [11]. These proteins are then targeted to either the cytoplasm or the mitochondria, depending on which translational start site is used [11]. Also, the deduced amino acid sequences of rat and human MCD are highly conserved, demonstrating approx. 90 % sequence identity. The greatest differences between MCDs from these two species are located in the N-terminus. Of the putative rat MCD phosphorylation sites identified previously [10], approximately half are missing from the human protein. The missing sites include Ser^{7}, Ser^{37}, Ser^{373}, Ser^{439} and Thr^{244}.

We have suggested that two start sites are potentially available for translation of the rat MCD cDNA sequence, and may be functional in transfected COS-7 cells (Figure 3A). However, by mutating the second putative start site for translation (Figure 3B), we demonstrated that the second methionine is not required to produce the 50.7 kDa isoform of MCD (Figure 3C). Furthermore, in rat liver only the 50.7 kDa isoform of the protein is present. This MCD isoform corresponds to the same molecular mass protein that was purified from the high-density fraction. Taken together, our data suggest that the more upstream methionine is the only start site necessary for translation of rat liver MCD, and that post-translational processing may account for the generation of the smaller 50.7 kDa isoform. It is still possible that either the control of these two potential start sites or post-translational processing may be a form of MCD regulation in different cell types. It appears that the pancreas contains both isoforms of MCD (Figure 4), suggesting that the larger of the two isoforms may play a specialized role in certain cell types. This is in agreement with our previous data showing that the full-length transcript is the predominant MCD transcript expressed in the β-cell [10]. In contrast with this, human fibroblasts and human heart tissue do not possess the full-length transcript [12], indicating that there are either differences between human and rat MCDs or differences between tissues. Furthermore, there is no evidence that two distinct transcriptional start sites exist, nor is there an indication of alternative splicing. However, it is possible that a full-length transcript is produced and that the first ATG may be removed by alternative splicing to produce the smaller MCD transcript. Transfection of a plasmid containing the rat liver MCD cDNA sequence with the first ATG deleted into COS-7 cells resulted in a 50.7 kDa MCD protein (results not shown). This indicates that the second ATG is functional as an initiating methionine. Taken together, we can conclude that at least two transcript lengths can be generated in mammalian cells, each of which may contain an ATG that is able to initiate translation. How the two transcript lengths are produced is still unknown. Of interest is the fact that no mammalian cell type has been shown to contain both sizes of MCD transcripts, providing further evidence to support the hypothesis that there may be tissue-specific processes involved in regulating MCD transcript length.

An alternative explanation for the existence of two MCD isoforms both in transfected COS cells and in the pancreas is that the 50.7 kDa isoform may be generated by the removal of the putative mitochondrial targeting pre-sequence of the 54.7 kDa isoform of MCD. This suggests that the only MCD protein that is translated is the one of molecular mass 54.7 kDa. Indeed, we have previously identified a pre-sequence and various cleavage sites in the N-terminus of the rat MCD cDNA sequence that may target MCD to the mitochondria and then allow for subsequent cleavage [10]. Transfection experiments indicate that over-expression of the 54.7 kDa isoform of MCD may overload the mechanism by which MCD is processed, resulting in both processed (50.7 kDa) and non-processed (54.7 kDa) forms of MCD. Our site-directed mutagenesis and transfection studies seem to suggest that this may, in fact, be the case. If N-terminal cleavage is the process by which two forms of MCD are generated, then the pancreas seems to be a specialized tissue in this regard; the reasons and/or mechanisms for this are not understood.

Using antibodies generated against purified MCD, we also showed that the levels of MCD are highest in tissues that oxidize fatty acids, such as the heart and liver (Figure 4, lanes 1 and 3). Similarly, the lowest levels of MCD are apparent in tissues with low fatty acid oxidation rates, the most dramatic example being the brain (Figure 4, lane 7). These results are in accordance with our previous work on MCD activity [10], indicating that MCD activity corresponds to MCD protein levels. These data are also consistent with MCD having a role in regulating fatty acid oxidation. Recently it has been shown that human heart and skeletal muscle possess the most abundant levels of MCD transcript [12]. This is in contrast with our previous data, which showed that rat skeletal muscle has a relatively low level of MCD activity as compared with liver or heart [10]. The data presented here may provide an explanation for why skeletal muscle MCD mRNA levels are high while activity is low. It appears that the level of MCD protein may be under translational regulation, such that MCD protein levels are relatively low even though MCD transcript levels are elevated. Alternatively, skeletal muscle may contain a mechanism that produces a more rapid turnover of MCD protein.

Although significant progress has been made with respect to the molecular characterization of MCD, much less is known about the physiological role of this enzyme. At this point only correlative data exist, which suggest that MCD is a regulator of fatty acid oxidation in both skeletal muscle and cardiac muscle [1,9]. In the liver, the regulation of malonyl-CoA levels is also an essential component in the control of fatty acid oxidation [24]. Since many enzymes that control lipid metabolism are altered in diabetes [25], we have studied MCD in two models that represent both Type I and Type II (insulin-resistant) diabetes. In 6-week-old streptozotocin-diabetic rats, many of the hepatic enzymes involved in the regulation of lipid metabolism are suppressed [17,26–28]. In these insulin-deficient animals, blood glucose levels and fatty acid levels rise [21], which was confirmed by our results (Table 2). Although clearly diabetic (i.e. elevated plasma fatty acids, dramatically reduced plasma insulin levels, and urine sugar levels $> 2\,\%$), these animals demonstrated only a modest level of hyperglycaemia. One explanation for this may be the dependence of plasma glucose levels on the feeding state of the animal and time of day when the animal was killed. Nevertheless,

in livers where glucose uptake is not dependent on insulin, the tissue is exposed to abnormally high levels of glucose [21]. The liver responds to this increase in glucose by inactivating ACC [5], resulting in a decrease in the production of malonyl-CoA. However, malonyl-CoA levels might remain elevated due to the associated down-regulation of fatty acid synthase [5]. Since malonyl-CoA levels are in fact decreased in livers from streptozotocin-diabetic rats [29], we propose that another mechanism regulates malonyl-CoA concentration. Given the rise in MCD activity in livers of streptozotocin-diabetic rats, we suggest that MCD is an important means by which malonyl-CoA levels are decreased in this model. One explanation for elevated MCD levels may be that MCD expression is related to transcriptional up-regulation upon exposure to elevated fatty acid levels. It is possible that the promoter region of MCD contains a fatty acid response element which is responsible for increased levels of MCD. Characterization of the promoter region of MCD will specifically address this possibility.

The *Cp*/*Cp* rat is deficient in the corpulent gene (*Cp*), and animals homozygous for *Cp* display insulin-resistance characterized by hypertriglyceridaemia, and hyperinsulinaemia with impaired glucose tolerance [14,20,22]. It has been shown that the white adipose tissue of *Cp*/*Cp* rats significantly overexpresses the obese (*ob*) gene mRNA as a result of a mutation in the leptin receptor [30]. It is now known that the *Cp*/*Cp* rats have no functional leptin receptors. In these obese animals, hepatic fatty acid synthesis is increased, as is ACC activity. Similarly, fatty acid synthase is still active, and more malonyl-CoA is directed towards fatty acid synthesis. Quite predictably, in livers from these animals we observed no significant change in MCD activity or expression (Figure 6). As a result, we hypothesize that MCD is not an important regulator of malonyl-CoA levels during insulin resistance.

Fasting and refeeding also have dramatic effects on fatty acid biosynthesis and oxidation [20]. During fasting, liver fatty acid oxidation becomes an important source of energy. Predictably, therefore, malonyl-CoA levels should decrease to preserve carnitine palmitoyltransferase 1 activity. As shown in Figure 7, we also observed a parallel increase in MCD activity during fasting. During refeeding of fasted rats, increases in both ACC and fatty acid synthase activities occur [4,5], and fatty acid oxidation decreases. Concomitant with this, we observed a significant decrease in MCD activity. Although MCD activity was altered during these various nutritional states, the level of MCD protein was not significantly altered (Figure 7). Dephosphorylation may account for the increase in MCD activity within groups; however, the increase in MCD activity in untreated livers from fasted rats compared with livers from control animals cannot be attributed to dephosphorylation. We propose that alternative regulatory mechanisms are involved under these conditions. Possible mechanisms that may also contribute to the regulation of MCD activity are the existence of a separate regulatory protein or allosteric regulation by another molecule. These possible regulatory mechanisms remain to be explored. Once again, this suggests that the activity of MCD is under post-translational regulation, only part of which, however, is through phosphorylation control.

Despite the recent advances in the cloning and characterization of MCD, there is still some controversy as to the exact subcellular localization of the protein. Recent reports have indicated that MCD may be localized to the cytoplasm, the mitochondria and/or the peroxisomes [1,10,12]. Regardless of the exact localization of MCD, however, it is now apparent that MCD is involved in regulating fatty acid oxidation, at least in heart and skeletal muscle [1,9]. Our data in liver also provide strong correlative evidence to suggest that MCD is involved in regulating fatty acid oxidation in the hepatocyte. Whether MCD is regulated by phosphorylation is an open question. The putative phosphorylation sites determined from the deduced amino acid sequence of MCD [10] are conserved between rat β-cell and rat liver MCD. We have not, however, been able to demonstrate phosphorylation *in vitro* using purified rat liver MCD. The activity of MCD was not altered *in vitro* by casein kinase II, protein kinase C or AMP-activated protein kinase, or by protein phosphatases 2A or 2C. This does not rule out the possibility that these enzymes act on MCD while not directly altering its activity. For example, phosphorylation by these kinases may be involved in other, unrelated, cellular mechanisms, such as control of the subcellular localization of MCD. Our studies did not assess these other possibilities.

In summary, we report the isolation, characterization and cloning of rat liver MCD. The purification of the protein confirms that its molecular mass, as predicted by the cDNA sequence, is 50.7 kDa. We have generated antibodies directed against MCD which demonstrate that the highest levels of MCD are found in oxidative tissues such as the liver and heart. Furthermore, we have shown that two forms of MCD exist (50.7 kDa and 54.7 kDa), one originating from the first translational start site present in the cDNA sequence and the other appearing to originate from post-translational processing of this larger isoform. To date, only the pancreatic β-cell of the rat has been shown to contain both forms of MCD, indicating that the post-translational processing may be regulated in specific cell types. Finally, we present evidence that MCD is involved in regulating fatty acid oxidation, supporting a growing number of publications suggesting a similar role for MCD [1,9].

We thank James C. Russell for providing us with the JCR:LA rats. This work was supported by grants from the Canadian Diabetes Association, the Medical Research Council of Canada and the Juvenile Diabetes Foundation. G.D.L. is a Medical Scientist of the Alberta Heritage Foundation for Medical Research. L.G.B. is a Medical Research Council of Canada and Alberta Heritage Foundation for Medical Research Scholar. M.P. is a Medical Research Council of Canada Scientist.

REFERENCES

1 Alam, N. and Saggerson, E. D. (1998) Biochem. J. **334**, 233–241
2 McGarry, J. D. and Brown, N. F. (1997) Eur. J. Biochem. **244**, 1–14
3 Louis, N. A. and Witters, L. A. (1992) J. Biol. Chem. **267**, 2287–2293
4 Kim, T. S. and Freake, H. C. (1996) J. Nutr. **126**, 611–617
5 Goodridge, A. G. (1991) in Biochemistry of Lipids, Lipoproteins and Membranes (Vance, D. and Vance, J., eds.), pp. 111–139, Elsevier, Amsterdam and New York
6 Bird, M. I. and Saggerson, E. D. (1984) Biochem. J. **222**, 639–647
7 Jang, S. H., Cheesbrough, T. M. and Kolattukudy, P. E. (1989) J. Biol. Chem. **264**, 3500–3505
8 Kolattukudy, P. E., Poulose, A. J. and Kim, Y. S. (1981) Methods Enzymol. **71C**, 150–163
9 Dyck, J. R., Barr, A. J., Barr, R. L., Kolattukudy, P. E. and Lopaschuk, G. D. (1998) Am. J. Physiol. **275**, H2122–H2129
10 Voilley, N., Roduit, R., Vicaretti, R., Bonny, C., Waeber, G., Dyck, J. R., Lopaschuk, G. D. and Prentki, M. (1999) Biochem. J. **340**, 213–217
11 Courchesne-Smith, C., Jang, S. H., Shi, Q., DeWille, J., Sasaki, G. and Kolattukudy, P. E. (1992) Arch. Biochem. Biophys. **298**, 576–586
12 Sacksteder, K. A., Morrell, J. C., Wanders, R. J., Matalon, R. and Gould, S. J. (1999) J. Biol. Chem. **274**, 24461–24468
13 Gao, J., Waber, L., Bennett, M. J., Gibson, K. M. and Cohen, J. C. (1999) J. Lipid Res. **40**, 178–182
14 Russell, J. C., Graham, S. and Hameed, M. (1994) Metab. Clin. Exp. **43**, 538–543
15 Sherwin, J. E. and Natelson, S. (1975) Clin. Chem. **21**, 230–234
16 Moir, A. M. and Zammit, V. A. (1994) Biochem. J. **304**, 177–182
17 Shillabeer, G., Hornford, J., Forden, J. M., Wong, N. C., Russell, J. C. and Lau, D. C. (1992) J. Lipid Res. **33**, 31–39
18 Witters, L. A., Gao, G., Kemp, B. E. and Quistorff, B. (1994) Arch. Biochem. Biophys. **308**, 413–419
19 Clarke, S. D., Armstrong, M. K. and Jump, D. B. (1990) J. Nutr. **120**, 218–224

20 Porter, J. W. and Swenson, T. L. (1983) Mol. Cell. Biochem. **53/54**, 307–325
21 Topping, D. L. and Targ, M. E. (1975) Horm. Res. **6**, 129–137
22 Mathe, D. (1995) Diabetes Metab. **21**, 106–111
23 Espeel, M. and Van Limbergen, G. (1995) J. Inherit. Metab. Dis. **18** (Suppl. 1), 135–154
24 McGarry, J. D., Takabayashi, Y. and Foster, D. W. (1978) J. Biol. Chem. **253**, 8294–8300
25 Miethke, H., Wittig, B., Nath, A. and Jungermann, K. (1986) Histochemistry **85**, 483–489
26 Lopaschuk, G. D. and Spafford, M. (1989) Circ. Res. **65**, 378–387
27 Katsurada, A., Iritani, N., Fukuda, H., Matsumura, Y., Nishimoto, N., Noguchi, T. and Tanaka, T. (1990) Eur. J. Biochem. **190**, 427–433
28 Ozcelikay, A. T., Becker, D. J., Ongemba, L. N., Pottier, A. M., Henquin, J. C. and Brichard, S. M. (1996) Am. J. Physiol. **270**, E344–E352
29 Elayan, I. M., Cartmill, D. C., Eckersell, C. B., Wilkin, J. and Winder, W. W. (1991) Proc. Soc. Exp. Biol. Med. **198**, 569–571
30 Vydelingum, S., Shillabeer, G., Hatch, G., Russell, J. C. and Lau, D. C. (1995) Biochem. Biophys. Res. Commun. **216**, 148–153

Received 30 September 1999/19 April 2000; accepted 12 June 2000