Conferral of Malonyl Coenzyme A Sensitivity to Purified Rat Heart Mitochondrial Carnitine Palmitoyltransferase†

Chang Ho Chung,‡ Gebre Woldegiorgis,§ Gonghe Dai,‡ Earl Shrago,§ and L. L. Bieber*,‡

Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824, and Departments of Medicine and Nutritional Science, University of Wisconsin, 430 Nutrition Science Building, 1415 Linden Drive, Madison, Wisconsin 53706

Received November 27, 1991; Revised Manuscript Received July 16, 1992

ABSTRACT: An immunoaffinity column against the 86-kDa malonyl-CoA-binding protein of beef heart mitochondria was prepared, and the properties of the eluates were compared to those of eluates of an anti-carnitine palmitoyltransferase immunoaffinity column. Both eluates contain seven to eight major proteins with a malonyl-CoA-binding capacity of approximately 5 nmol/mg of protein; in contrast, the eluates from a preimmune IgG column did not contain any of the major proteins. The eluates from both immunoaffinity columns conferred malonyl-CoA sensitivity to purified rat heart mitochondrial carnitine palmitoyltransferase (CPT_i/CPT-II). Addition of phospholipids increased the degree of malonyl-CoA inhibition. Doubling the amount of column eluate approximately doubled the malonyl-CoA sensitivity when added to a fixed amount of CPT; i.e., the inhibition increased from 32 to 67%. These results show that CPT_i/CPT-II is capable of exhibiting malonyl-CoA sensitivity in the presence of malonyl-CoA-binding proteins. The results do not support the concept that the 86-kDa malonyl-CoA-binding protein is detergent-inactivated carnitine palmitoyltransferase I; rather, they suggest that it is a regulatory subunit of a carnitine palmitoyltransferase complex.

It is well established that carnitine palmitoyltransferase (CPT),[1] is required for the mitochondrial β-oxidation of long-chain fatty acids. This function of mitochondrial CPT requires one form of the enzyme to be in contact with the cytosolic milieu and the other form to be in contact with the mitochondrial matrix (Bieber, 1988). During the past 2–3 decades, considerable efforts have been devoted toward establishing the precise location of the two forms of CPT (McGarry et al., 1989) and determining whether different catalytic proteins are responsible for the activity in contact with the matrix compartment of mitochondria versus that which interacts with cytosolic substrates. The fact that the form in contact with the cytosolic compartment, CPT-I/CPT_o, can be inhibited by malonyl-CoA (McGarry et al., 1978; Declercq et al., 1987) and the fact that this form of the enzyme may be associated with the outer, rather than the inner, membrane of mitochondria (Murthy & Pande, 1987) have caused many to conclude that CPT_o and CPT_i (the form in contact with the matrix) are different proteins. However, studies in this area are complicated by the fact that liver contains at least three enzymes which exhibit CPT activity. In addition to a mitochondrial CPT, another enzyme is associated with peroxisomes, and another is associated with microsomes. All three enzymes have the capacity to convert both medium-chain and long-chain acyl-CoAs to acylcarnitines (Bieber, 1991; Bieber et al., 1991), and both mitochondrial CPT activity and the microsomal long-chain carnitine acyltransferase activity are inhibited by malonyl-CoA (Lilly et al., 1990).

Studies with heart mitochondria show that a malonyl-CoA-sensitive CPT can be isolated as part of a complex that contains the enzymes of β-oxidation, indicating malonyl-CoA-sensitive CPT may be in contact with the mitochondrial β-oxidation enzymes (Kerner & Bieber, 1990). This complex contains a single molecular weight species of CPT. Woldegiorgis et al. (1990, 1992) have recently presented evidence that the malonyl-CoA-binding protein [this 86-kDa protein is considered by some to be a detergent-labile malonyl-CoA-sensitive CPT (McGarry et al., 1978, 1989; Declercq et al., 1987)] can confer malonyl-CoA sensitivity to impure preparations of malonyl-CoA-insensitive CPT, and Saggerson and colleagues (Ghadiminejad & Saggerson, 1990a,b, 1991) also have presented data which show that when the CPT activity of liver mitochondria is separated from the malonyl-CoA-binding proteins, loss of malonyl-CoA sensitivity occurs, but malonyl-CoA sensitivity can be restored by addition of the malonyl-CoA-binding fraction to impure preparations of CPT. Such studies indicate that the sensitivity of CPT to malonyl-CoA is due to the presence of a malonyl-CoA-binding protein, possibly a regulator protein. Herein we show that when fractions containing partially purified malonyl-CoA-binding protein are added to purified CPT in the presence of phospholipids, malonyl-CoA sensitivity is conferred to purified rat heart mitochondria CPT_i.

MATERIALS AND METHODS

Preparation of Rat and Beef Heart Mitochondria. Rat heart mitochondria were isolated from Sprague-Dawley rats weighing between 100 and 200 g as described (Kerner & Bieber, 1990) and then were suspended at a concentration of 40 mg of protein/mL in 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, and 5 mM Tris, pH 7.5. They were either used immediately or stored at −70 °C. Beef heart mitochondria were prepared according to the procedure of Green et al. (1957).

Purification of CPT and of the 86 000-Dalton Malonyl-CoA-Binding Protein. CPT was purified from rat heart

† Supported by NIH Grants DK 18427 and GM 14033. G.W. received a Martin Luther King/Rosa Parks/Ceasar Chavez fellowship from Michigan State University.

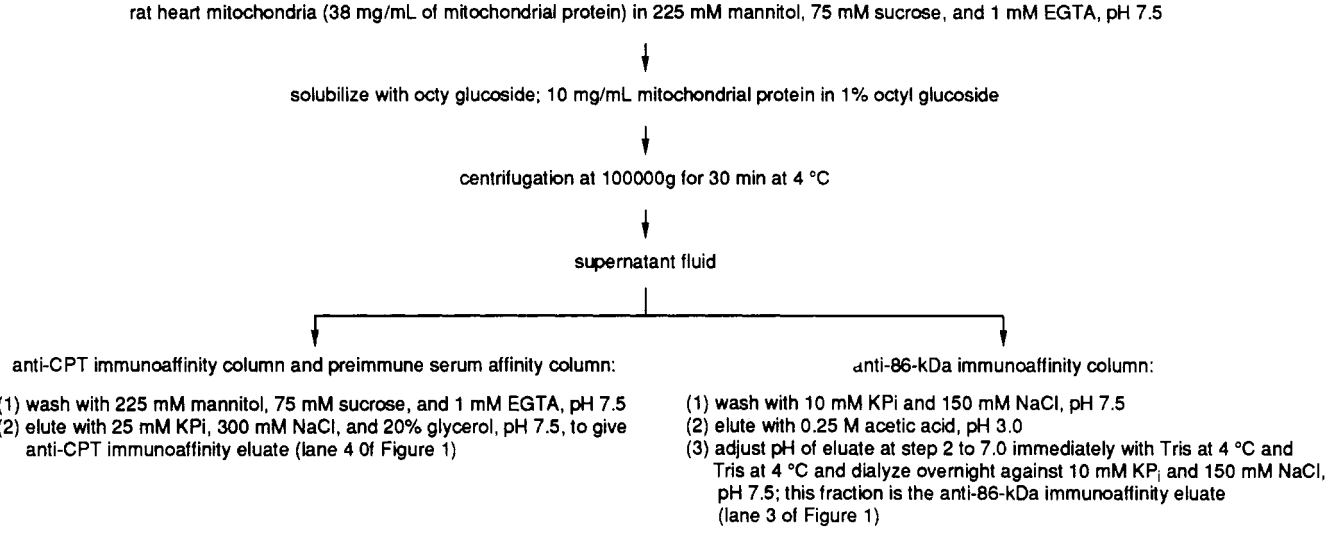
‡ Michigan State University.

§ University of Wisconsin.

[1] Abbreviations: CPT, carnitine palmitoyltransferase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.

0006-2960/92/0431-9777$03.00/0

Chart I: Flow Diagram for the Separation of Proteins Retained by Immunoaffinity Chromatography[a]



[a] These antibody column eluates were used in the studies to confer malonyl-CoA sensitivity to purified CPT.

mitochondria as described previously (Kerner & Bieber, 1990), and the 86-kDa malonyl-CoA-binding protein from beef heart mitochondria was purified as described by Woldegiorgis et al. (1992).

Gel electrophoresis of the fractions and of octyl glucoside extracts of beef heart mitochondria was performed on 15% polyacrylamide–SDS slab gels according to Laemmli (1970). The 86-kDa protein bands were excised from the gels and the gel slices rinsed with deionized water. The protein was then electroeluted from the gel slices with a buffer containing 25 mM Tris, 192 mM glycine, and 1% SDS, using a Bio-Rad electroeluter apparatus according to the manufacturer's instructions. The protein was precipitated by the addition of 9 volumes of 100% ethanol at −20 °C for 24 h and recovered by centrifugation at 15 000 rpm for 20 min. The pellet was resuspended in 1 mL of 0.1% SDS and reprecipitated with 3 volumes of 100% ethanol; this procedure was repeated 3 times. The final pellet was dissolved in 1.5 mL of 0.1% SDS, dialyzed against 0.1% SDS, and then lyophilized.

Generation of Immune Serum. Preimmune serum was collected prior to primary immunization for each of the two antigens. Polyclonal anti-CPT antibody was prepared and purified as described by Kerner and Bieber (1990). Polyclonal anti-86-kDa antibody was prepared by injecting 200 μg of electroeluted 86-kDa protein in 100 μL of 5% SDS which had been diluted with 900 μL of 150 mM sodium chloride/150 mM sodium phosphate, pH 7.5. This solution was mixed with an equal volume of complete Freund's adjuvant by repeated passage through a needle and injected into rabbits by multiple subcutaneous, intradermic injections. After 3 weeks, each rabbit was boosted by subcutaneous injection of 200 μg of protein in an incomplete Freund's adjuvant emulsion. The booster injections were repeated twice at 3-week intervals, and 3 weeks after the last injection, three booster injections were administered subcutaneously every other day. Ten days after the last booster injection, blood was obtained from an ear vein. Antisera were decomplemented by treatment at 56 °C for 30 min (Brandolin et al., 1989). Rabbits were bled before each booster injection, and the presence of antisera to the 86-kDa malonyl-CoA-binding protein was determined by an ELISA method using polystyrene microtitration plates from NUNC as described by Brandolin et al. (1989).

The immunoglobulin fraction from rabbit antiserum was purified using a Gamma Bind G-prepak cartridge from GENEX with the protocol supplied by the company. After elution from the Gamma Bind G column, IgG was used immediately or stored at −70 °C in 150 mM sodium chloride/25 mM potassium phosphate, pH 7.5. The anti-malonyl-CoA-binding immunoaffinity column was prepared as described by Harlow and Lane (1988), and the anti-CPT immunoaffinity column and the preimmune serum affinity columns were prepared as described (Kerner & Bieber, 1990).

Application to and Elution of Proteins from the Immunoaffinity Columns. Freeze-thawed mitochondria in 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, and 5 mM Tris, pH 7.5, were diluted with the buffer to a concentration of 10 mg/mL and made 1% in octyl glucoside. After standing for 30 min on ice with frequent shaking, the mitochondrial extracts were centrifuged for 30 min at 100000*g* and the supernatant fluids removed. The pellet was reextracted as described above, and aliquots of the combined supernatant fluids were loaded onto the immunoaffinity columns which had been preequilibrated with 25 mM potassium phosphate, pH 7.5, in 150 mM NaCl. For the CPT immunoaffinity column and the preimmune serum affinity column, unbound proteins were removed by washing with the mannitol/sucrose buffer described above until the absorbance at 280 nm was less than 0.01. Then the column was eluted with 20% glycerol, 300 mM sodium chloride, and 25 mM potassium phosphate, pH 7.5, to remove β-oxidation complex enzymes as described [see Figure 6 of Kerner and Bieber (1990)]. This fraction, containing the malonyl-CoA-binding proteins and β-oxidation enzymes, was concentrated and dialyzed against 25 mM potassium phosphate, pH 7.5, buffer containing 20% glycerol. The procedure was the same for the 86-kDa malonyl-CoA-binding protein immunoaffinity column chromatography, except unbound proteins were removed by washing with 150 mM sodium chloride/25 mM potassium phospahte, pH 7.5. Bound proteins were then eluted with 0.25 M acetic acid, pH 3.0. The eluted proteins were immediately adjusted to pH 7.0 with Tris at 4 °C, concentrated, and dialyzed overnight against 150 mM sodium chloride/10 mM potassium phosphate, pH 7.5. The antibody column separations are summarized in the flow diagram shown in Chart I.

Preparation of Liposomes. Three hundred microliters of phosphatidylcholine (10 mg/mL), 240 μL of phosphatidylethanolamine (10 mg/mL), and 120 μL of cardiolipin (5 mg/mL) in chloroform were dried under nitrogen in a preweighed

tube. After being dried overnight in vacuum, the tubes were weighed, flushed with nitrogen, and sonicated in 0.1 M potassium phosphate containing 0.1 mM EDTA at pH 7.5. This phospholipid mixture was used for the reconstitution studies.

Assays. CPT was determined by the isotope forward assay essentially as described (Fiol et al., 1987). The assay mixture contained 120 mM KCl, 20 mM sucrose, 10 mM Tris, pH 7.5, 1 mM EGTA, 10 mM L-carnitine, and 17 μM [1-^{14}C]decanoyl-CoA with or without 50 μM malonyl-CoA in a final volume of 100 μL. The reaction was initiated by adding purified CPT (5 μL) and stopped by addition of 400 μL of cold methanol, and the radioactivity in the decanoylcarnitine was determined (Fiol et al., 1987). Protein was determined by a modification of the Lowry method (Markwell et al., 1981). Malonyl-CoA binding in the immunoaffinity column eluates was determined by the method of Penefsky (1977), using 4.4 μM [2-^{14}C]malonyl-CoA with a specific activity of 55 mCi/mmol. SDS–PAGE was performed using 12% slab gels according to the procedure of Laemmli (1970). After electrophoresis, the gel was blotted onto a poly(vinylidene difluoride) membrane. The purified 86-kDa and CPT antisera were diluted 100-fold and 2000-fold, respectively, in 50 mM Tris buffer, pH 7.5, containing 250 mM sodium chloride and 1% fatty acid-free bovine serum albumin. For Western blots, anti-rabbit IgG antibodies conjugated to alkaline phosphatase were used according to instructions provided by Bio-Rad. For autoradiography, gel electrophoresis was carried out on 15% polyacrylamide–SDS slab gels, and the proteins were electrophoretically transferred from the slab gel to the nitrocellulose sheet using a Bio-Rad immunoblot apparatus. The nitrocellulose membranes were blocked with 3% albumin for 1 h at room temperature and incubated overnight at 4 °C with the rabbit antiserum diluted 50-fold in the saline albumin buffer described above. After three washings with this buffer, containing 1% Nonidet P40, the filter was then incubated for 2 h at room temperature with 2 μCi of [^{125}I]protein G recombinant (Gamma Bind 2, GENEX Corp.) in the 20-mL saline buffer/bovine serum albumin mixture described above. After four washes with saline/albumin as described above, the nitrocellulose membrane was dried and processed for autoradiography.

Reconstitution of Malonyl-CoA Sensitivity. In most studies, 5 μL of purified CPT containing 1.9 μg of protein was incubated with 45 μL of anti-CPT antibody column eluate, approximately 35 μg of protein, for 1 h at 4 °C. The solution was divided equally, 25 μL, into two Eppendorf tubes. To one was added 33 μL of 0.1 M potassium phosphate, pH 7.5, and 0.1 mM EDTA. To the other was added 33 μL (300 μg) of the sonicated phospholipid mixture containing phosphatidylcholine/phosphatidylethanolamine and cardiolipin with a weight ratio of 5:4:1 in 0.1 mM potassium phosphate buffer (pH 7.5)/0.1 mM EDTA. The samples were incubated for 10 min at 4 °C, frozen for 30 min at –70 °C, thawed at 30 °C, and then sonicated for 10 s at 4 °C in a heat systems cup sonicator at a setting of 10. Equal aliquots of these mixtures were assayed for CPT activity in the absence and presence of 50 μM malonyl-CoA. The procedure was essentially the same for the experiments in which the immunoaffinity eluates (35–40 μg of protein) from the anti-86-kDa malonyl-Co-A-binding affinity column were used with 1.9 μg of purified CPT.

RESULTS

Partial Purification of Beef Heart Mitochondrial Malonyl-CoA-Binding Protein. The malonyl-Co-A-binding protein from heart mitochondria was purified as described for rat liver mitochondria (Woldegiogris et al., 1992). Chromatography on hydroxyapatite showed three peaks of malonyl-CoA binding (data not shown). The maximum malonyl-CoA-binding capacity was approximately 1400 pmol/mg of protein, which is about 22-fold greater than that obtained for solubilized beef heart mitochondria. The SDS–PAGE pattern of the purified malonyl-CoA-binding fraction showed three protein bands corresponding to molecular masses of 86, 45, and 33 kDa (data not shown). The 86-kDa band was electroeluted for antibody preparation.

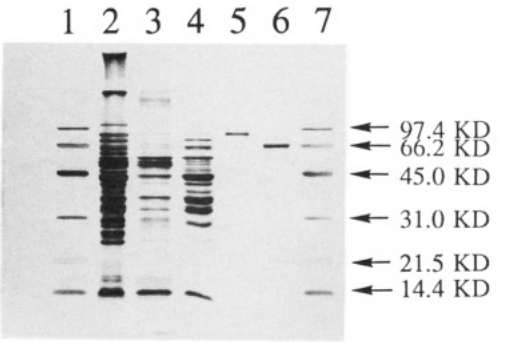


FIGURE 1: SDS–polyacrylamide gel electrophoresis of the eluates from immunoaffinity chromatography of octyl glucoside solubilized heart mitochondria. Lanes 1 and 7, standard molecular mass markers: phosphorylase *b* (97 400 Da), bovine serum albumin (66 200 Da), ovalbumin (45 000 Da), carbonic anhydrase (31 000 Da), trypsin inhibitor (21 500 Da), and lysozyme (14 400 Da), indicated by the arrows from top to bottom, respectively. Lane 2, 1% octyl glucoside solubilized extract of rat heart mitochondria; lane 3, 7 μg of the anti-86-kDa eluate; lane 4, 10 μg of anti-CPT eluate; lane 5, purified 86-kDa malonyl-CoA-binding protein, 0.7 μg; lane 6, purified rat heart mitochondrial CPT, 1.0 μg. The gel was silver-stained.

Characterization of the Anti-Malonyl-CoA-Binding Protein Immunoaffinity Column Eluates. It was previously shown that anti-CPT column eluates containing malonyl-CoA-binding proteins can confer malonyl-CoA sensitivity to the CPT isolated from an anti-CPT affinity column; however, the degree of inhibition varied between 0 and 40% (Kerner & Bieber, 1990). Since a high molecular weight malonyl-CoA-binding protein is present in the anti-CPT antibody column eluates, it was decided to make an immunoaffinity column using the antibody against the 86-kDa malonyl-CoA-binding protein. Figure 1 shows the SDS–PAGE profile of the purified 86-kDa protein, lane 5, and purified rat heart CPT in lane 6. The protein from lane 5 was used for immunization and subsequent IgG preparation. A Western blot, using this antibody against an octyl glucoside solubilized preparation of beef heart mitochondria, is shown in Figure 2. In addition to a strong band at 86 kDa, weaker interactions occur at 40 and 26 kDa. This antibody was used for preparation of an immunoaffinity column, and octyl glucoside extracts of rat heart mitochondria, identical to those used previously with the anti-CPT immunoaffinity column (Kerner & Bieber, 1990), were passed both through the immunoaffinity column against the 86-kDa protein and through the immunoaffinity column against CPT, as described under Materials and Methods. Lane 3 of Figure 1 shows the profile of the proteins eluted from the immunoaffinity column against the 86-kDa protein, and lane 4 shows the protein profile of the anti-CPT immunoaffinity column. This latter eluate is equivalent to fraction A-1 of Figure 6 previously published (Kerner & Bieber, 1990). It contains three of the four mitochondrial β-oxidation enzymes, as well as malonyl-CoA-binding proteins, but the eluate is devoid of CPT activity. It also contains carnitine/acylcarnitine translocase activity (Murthy & Bieber,

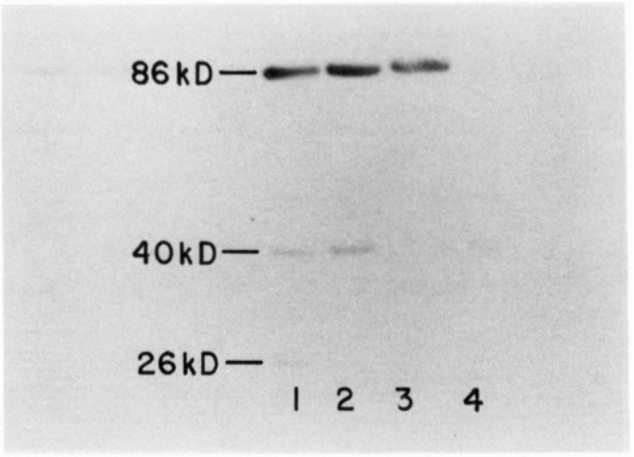


FIGURE 2: Western blot analysis of beef heart and rat liver mitochondrial preparations using anti-86-kDa malonyl-CoA-binding protein sera. Lysed mitochondria, detergent extracts, and purified malonyl-CoA-binding protein fractions were electrophoresed on a 15% slab gel and electroblotted on a poly(vinylidene difluoride) membrane. After being blocked with 3% BSA, the membrane was incubated with the anti-86-kDa sera as outlined under Materials and Methods, followed by incubation with 2 μCi of ^{125}I-labeled protein G, recombinant (Gamma Bind-2, GENEX Corp.), for 2 h at room temperature: (1) lysed beef heart mitochondria, 225 μg; (2) octyl glucoside extract, beef heart mitochondria, 98 μg; (3) purified beef heart mitochondria 86-kDa malonyl-CoA-binding protein; (4) rat liver mitochondria, 100 μg.

Table I: Malonyl-CoA Binding by the Immunoaffinity Column Eluates of Rat Heart Mitochondria[a]

preparations	malonyl-CoA bound (nmol/mg of protein)
anti-CPT immunoaffinity eluate	4.5
anti-86-kDa immunoaffinity eluate	5.3

[a] Rat heart mitochondria were solubilized in octyl glucoside as described under Materials and Methods. The values represent an average of two experiments; $n = 2$.

1991). The eluates from both immunoaffinity columns contain seven to eight proteins with similar, if not identical, molecular masses, including a weak band at 86 kDa. The malonyl-CoA-binding capacity of both immunoaffinity eluates is given in Table I; both have a binding capacity of approximately 5 mmol of malonyl-CoA/mg of protein.

Studies on Restoration of Malonyl-CoA Sensitivity to Purified CPT. Experiments were done to determine the effect of the immunoaffinity column eluates, the proteins shown in lanes 3 and 4 of Figure 1, on the malonyl-CoA sensitivity of purified CPT. Two problems became evident during the initial experiments. When protein was added to purified CPT, the activity increased. For example, addition of 70 μg of antibody column eluate from the anti-86-KDa protein to 1.9 μg of CPT increased CPT activity approximately 2-fold. This could either be due to an effect of the added protein on the V_{max} of purified CPT or be due to activation of a latent CPT in the antibody column eluate. This was investigated by adding different amounts of bovine serum albumin to purified CPT. The results are shown in Table II. Addition of 25 μg of albumin to 1.9 μg of CPT more than doubled the CPT activity. Thus, addition of protein to purified preparations of CPT increases the V_{max} of the enzyme. For subsequent experiments, the control CPT is the activity obtained in the presence of the added protein.

A large variation in the degree of malonyl-CoA sensitivity occurred when the immunoaffinity eluates were added to purified CPT; some preparations gave as high as 40% inhibition, while others were lower and some were 0. Since

Table II: Effect of Bovine Serum Albumin on CPT from Rat Heart Mitochondria[a]

bovine serum albumin (μg)	nmol of decanoylcarnitine min^{-1} (μg of CPT)$^{-1}$
0	99
10	160
15	228
25	241

[a] The values represent an average of two experiments; $n = 2$; 1.9 μg of rat heart mitochondrial CPT was incubated for 5 min at 4 °C with albumin and then assayed for CPT.

Table III: Effect of Different Combinations of Phospholipids on Malonyl-CoA-Sensitive Purified CPT from Rat Heart Mitochondria[a]

PC:PE:CL ratio	μg/μL	nmol of decanoylcarnitine min^{-1} (μg of CPT)$^{-1}$ −50 μM malonyl-CoA	nmol of decanoylcarnitine min^{-1} (μg of CPT)$^{-1}$ +50 μM malonyl-CoA	% inhibition by malonyl-CoA
5:4:1	10.3	241	144	40
0:4:1	5.1	206	149	27
5:4:0	9.3	294	200	32
5:0:1	6.2	248	173	30
5:0:0	5.1	334	238	29
0:4:0	4.1	337	251	26
0:0:1	1.0	271	238	12
0	0	126	101	25

[a] Numbers represent an average of two experiments; $n = 2$. Forty micrograms of the 86-kDa immunoaffinity chromatography eluate was preincubated with 1.9 μg of purified rat heart mitochondrial CPT for 1 h at 4 °C and treated as described under Materials and Methods. Total volume = 58 μL. Phosphatidylcholine (PC):phosphatidylethanolamine (PE):cardiolipin (CL) (w/w/w) ratio.

phospholipids can form vesicles or membrane structures that might be involved in malonyl-CoA sensitivity, the effect of different mixtures of phospholipid on the conferral of malonyl-CoA sensitivity to CPT was determined. The data in Table III show that the most effective combination of phospholipids tested is a ratio of 5:4:1 of phosphatidylcholine/phosphatidylethanolamine/cardiolipin at a concentration of 10.3 μg/μL. As shown in Table III, phospholipids also effect the activity of purified CPT in the presence of immunoaffinity column extracts. For all reconstitution experiments, control CPT activity was the activity in the presence of both phospholipids and added protein. For all reconstitution experiments reported herein, this ratio of phosphatidylcholine/phosphatidylethanolamine/cardiolipin was used.

The effect of the addition of anti-CPT and anti-86-kDa immunoaffinity eluates to purified CPT in the presence of phospholipids is shown in Table IV. Addition of 30–40 μg of antibody column eluate to 1.8 μg of purified CPT gave an average of 31% inhibition by 50 μM malonyl-CoA. Although the results in Table IV represent data from three experiments, a total of six experiments were done in which the immunoaffinity column eluates for anti-CPT were added to purified CPT using the conditions described for Table IV. An average of 31.6% ± 5.6% inhibition of purified CPT by 50 μM malonyl-CoA was obtained. Since a fixed concentration of antibody column eluates was used for these experiments, it was not apparent whether 31% inhibition represents the maximum inhibition of CPT or whether increasing the amount of antibody column eluate could increase the degree of inhibition. Some preliminary studies had shown malonyl-CoA inhibition in excess of 50%. The effect of doubling the amount of anti-86-kDa immunoaffinity eluate on inhibition of CPT by malonyl-CoA is shown in Figure 4. Although only two experiments were performed, the results show that doubling

Table IV: Effect of Immunoaffinity Column Eluates on Malonyl-CoA Sensitivity of Purified Rat Heart Mitochondrial CPT[a]

	nmol of decanoylcarnitine min^{-1} (mg of protein)$^{-1}$					
	−phospholipids			+phospholipids		
preparations	−50 μM malonyl-CoA	+50 μM malonyl-CoA	% inhibition	−50 μM malonyl-CoA	+50 μM malonyl-CoA	% inhibition
CPT	108.7 ± 5.8	113.3 ± 12.3	0	133.8 ± 38.8	125.8 ± 20.2	6
CPT + anti-86-kDa eluate	123.0 ± 1.0	114.9 ± 22.4	6	280.7 ± 3.7	193.4 ± 24.1	31
CPT + anti-CPT eluate	186.2 ± 17.8	179.9 ± 9.9	3	277.2 ± 10.1	190.0 ± 4.4	31
anti-86-kDa eluate	3.7 ± 0.2	3.5 ± 0.1	0	4.5 ± 0.3	4.2 ± 0.2	0
anti-CPT eluate	1.5 ± 0.1	1.5 ± 0.1	0	2.1 ± 0.2	5.3 ± 0.4	0

[a] Thirty-five to forty micrograms of the immunoaffinity eluates was preincubated with 1.8 μg of purified carnitine palmitoyltransferase for 1 h at 4 °C and 10.3 μg/μL phospholipids (PC:PE:CL weight ratio of 5:4:1) in 58 μL as described under Materials and Methods. For the control, 0.1 M potassium phosphate and 0.1 mM EDTA, pH 7.5, were used. Aliquots (5 μL) of the mixtures were assayed for the formation of decanoylcarnitine in the absence or presence of 50 μM malonyl-CoA. Results represent mean ± SEM, $n = 3$.

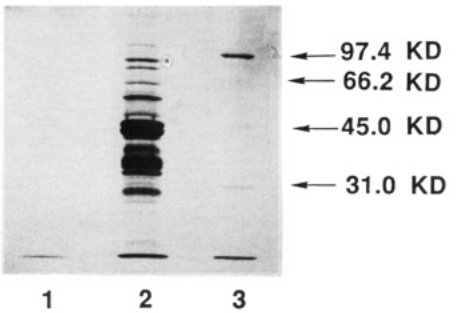


FIGURE 3: Comparison of the SDS–PAGE protein profiles of eluates from a preimmune serum affinity column to the profiles of anti-CPT immunoaffinity eluates of octyl glucoside solubilized rat heart mitochondria. 40.1 mg of IgG from preimmune serum and 43.2 mg of IgG from anti-CPT serum, purified with protein G, were coupled to Sepharose 4B by the method of Kerner and Bieber (1990), and 74 mg of solubilized mitochondria was passed over each column. The fractions equivalent to A1 of Figure 6 for Kerner and Bieber (1990) were concentrated and run on 12% SDS–PAGE. Lane 1 is the preimmune serum column fraction. It was 33 times more concentrated than the anti-CPT immunoaffinity column eluate shown in lane 2. The asterisk indicates the 86 000-kDa protein. Lane 3 represents the molecular mass markers.

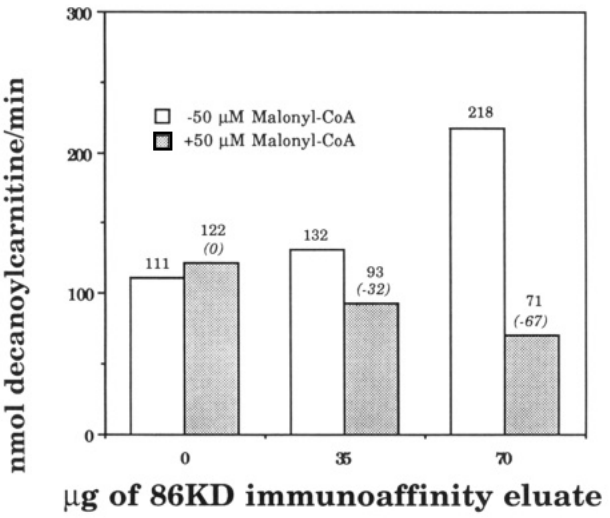


FIGURE 4: Effect of doubling the amount of the 86-kDa immunoaffinity chromatography eluate on malonyl-CoA sensitivity of purified CPT. Aliquots of the 86-kDa eluate were incubated with 1.7 μg of purified rat heart mitochondrial CPT for 1 h at 4 °C with 300 μg of phospholipids in 58 μL. The data are an average of two experiments, and the numbers in parentheses represent the percent inhibition of CPT by 50 μM malonyl-CoA. The open bar and the closed bar are with and without 50 μM malonyl-CoA, respectively.

the amount of immunoaffinity eluate almost doubled the degree of inhibition by malonyl-CoA. The average percent inhibition increased from 32% for 35 μg of eluate to 67% when 70 μg of eluate was added. Increasing the amount of eluate from 35 to 70 μg of protein also increased CPT activity from 132 to 218 nmol of decanoyl-CoA formed, consistent with the data shown in Table II.

Some of the experiments were repeated using antibody prepared against a new batch of purified rat heart mitochondrial CPT. The SDS–PAGE profile of the fraction, equivalent to fraction A1 of Kerner and Bieber (1990) for the new CPT antibody column and a similar column prepared from preimmune serum, is shown in Figure 3; although the anti-CPT profiles of Figures 2 and 3 are similar, in contrast, the preimmune serum column eluate was essentially protein-free. Some of the experiments described in Table IV were also repeated using the new preparation of CPT and the antibody eluates from the new anti-CPT immunoaffinity column. An average of 36.1 ± 19.7% inhibition ($n = 7$) was obtained when CPT, phospholipid, and antibody column eluate were used, and an average of 12% inhibition was obtained in the absence of phospholipids.

DISCUSSION

Conferral of Malonyl-CoA Sensitivity to CPT. The results confirm and extend the preliminary report (Chung et al., 1991) that malonyl-CoA sensitivity can be conferred to purified rat heart mitochondrial CPT by addition of an anti-CPT immunoaffinity eluate. This eluate contains malonyl-CoA-binding proteins, including the malonyl-CoA-binding protein studied intensively by others (McGarry et al., 1978, 1989; Declercq et al., 1987; Woldegiorgis et al., 1990; Ghadiminejad & Saggerson, 1990a,b, 1991; Murthy & Pande, 1990; Lund, 1987). When similar experiments were performed using the antibody prepared against a purified malonyl-CoA-binding protein, the eluates from this immunoaffinity column gave SDS-PAGE protein profiles similar to the anti-CPT eluates (compare lanes 3 and 4 of Figure 1). Thus, the protein profiles for the anti-malonyl-CoA-binding immunoaffinity column eluates are very similar to the anti-CPT immunoaffinity column eluates. We previously demonstrated that the anti-CPT column eluates contain the carnitine acylcarnitine translocase (Murthy & Bieber, 1991) and three of the four enzymes of the β-oxidation complex but lack of the flavin-linked acyl-CoA dehydrogenase (Kerner & Bieber, 1990). Although the malonyl-CoA-binding protein of rat heart mitochondria appears to be associated with a complex that contains some of the β-oxidation enzymes, as well as CPT, the amount of CPT associated with the anti-CPT column eluates and the total amount of 86-kDa protein associated with the anti-malonyl-CoA-binding protein column eluates are a small portion of the total protein. From SDS–PAGE profiles, the intensities of each of these proteins appear to represent less

than 5% of the total protein. If the proteins in the immunoaffinity eluates represent a functional complex which includes CPT, the 86-kDa malonyl-CoA-binding protein, and some of the enzymes of β-oxidation, it may not be surprising that CPT and the 86-kDa protein are quantitatively minor components. The enzymes of β-oxidation, particularly the hydroxyacyl-CoA dehydrogenase and the hydratase activities, are a family of enzymes, each of which contains multiple subunits (Bennett, 1990); consequently, the amount of protein contributed by the β-oxidation enzyme subunits should be larger.

The data in Figure 4 imply that a stoichiometry exists between the degree of malonyl-CoA inhibition of purified CPT and the amount of 86-kDa malonyl-CoA-binding protein, since doubling the amount of anti-malonyl-CoA-binding protein column eluate doubled the percent inhibition of a fixed amount of purified CPT by malonyl-CoA.

Although the data do not prove that the 86-kDa malonyl-CoA-binding protein is a regulatory protein, the fact that the preparations which confer malonyl-CoA sensitivity contain an 86 000-dalton protein strongly implies that it confers malonyl-CoA sensitivity to CPT. To date, we have not obtained complete inhibition either of purified CPT or of impure preparations of CPT by addition of extracts containing the malonyl-CoA-binding protein. The investigations have been hampered by the limited availability of the malonyl-CoA-binding protein and the lack of a specific assay to determine whether the malonyl-CoA-binding protein has been isolated in a functional form. The literature describes many studies which show that malonyl-CoA sensitivity can be lost in intact mitochondria. Although the causes for the loss of malonyl-CoA sensitivity are not known, several possibilities exist. These include the possibility that the protein is easily altered or denatured, causing it to lose its capacity to confer malonyl-CoA sensitivity to CPT. It is known that changes in membrane fluidity can alter the sensitivity of CPT-I/CPT_o to malonyl-CoA (Kolodziej & Zammit, 1991) and that detergents can affect the binding capacity of malonyl-CoA-binding proteins. Loss of malonyl-CoA sensitivity could also be due to dissociation of the malonyl-CoA-binding protein from catalytic CPT as discussed below, or loss of phospholipids. Regardless, our results agree with other reports (Woldegiorgis et al., 1990; Ghadiminejad & Saggerson, 1990a,b, 1991; Lilly et al., 1992) which show the malonyl-CoA-binding components can be separated from catalytically active CPT and that malonyl-CoA sensitivity can be restored by combining the separated components.

Ambiguity in the Location of Malonyl-CoA-Sensitive CPT. The anti-CPT column eluates and the anti-malonyl-CoA-binding protein column eluates contain similar proteins. Both eluates contain the 68 000-dalton CPT agreed by most, if not all, investigators in the field to be CPT-II/CPT_i, the form of CPT in contact with the mitochondrial matrix. Thus, this form of CPT is associated with the inner portion of the inner mitochondrial membrane. Of the proteins that have been identified in the anti-CPT immunoaffinity column eluates (CPT, the carnitine/acylcarnitine translocase, three of the four enzymes of β-oxidation, and the malonyl-CoA-binding component), at least five must be associated with the inner membrane of mitochondria. The location of the malonyl-CoA-binding protein is less certain. Thus, the preparations used herein appear to come from the inner membrane of mitochondria. Considerable evidence has accumulated that membrane fractions isolated by differential centrifugation, which are enriched in the outer membrane of liver mitochondria, contain malonyl-CoA-inhibitable CPT activity (Murthy & Pande, 1987, 1991; Ghadiminejad & Saggerson, 1990a,b). The recent report of Hoppel et al. (1992) is pertinent to this issue. Rat liver mitochondrial fractions were separated after disruption using a French press. The membrane fraction containing the outer mitochondrial membrane marker monoamine oxidase was essentially devoid of malonyl-CoA-inhibitable CPT. Three of the four mitochondrial membrane disruption techniques used did not support an outer membrane location for CPT; consequently, it was concluded that the outer membrane of liver mitochondria does not contan malonyl-CoA-sensitive CPT activity (Hoppel, 1991). In contrast, topographical studies using intact mitochondria and proteases indicate an outer membrane location for CPT_o and for malonyl-CoA-binding proteins (Kashfi & Cook, 1991; Murthy & Pande, 1987). Clearly, additional studies are required to resolve this issue.

Very recently, Kolodziej and colleagues (Kolodziej et al., 1992) reported that antibody against the 86 000-dalton malonyl-CoA-binding protein precipitates malonyl-CoA-sensitive CPT_o of outer mitochondrial-enriched membrane fractions but this antibody did not show a Western blot against purified CPT_i. This was interpreted as evidence for the existence of two different catalytic CPT proteins in mitochondria. However, the data in Figure 4 show that the antibody against the 86 000-dalton malonyl-CoA-binding protein (presumably the same protein as the one studied by Kolodziej) also retains CPT_i on immunoaffinity column chromatography and, thus, should also precipitate it. Unfortunately, the results of Western blots using anti-CPT antibody probes against the material precipitated by the anti-86 000-dalton antibody were not reported. Our results indicate that antibody-precipitated malonyl-CoA-sensitive CPT will show the presence of cross-reactive material against anti-CPT_i on Western blotting. Experiments are in progress to test this.

Number of Catalytic CPT Proteins in Mitochondria. The fact that addition of malonyl-CoA-binding proteins to purified rat heart mitochondrial CPT (CPT_i/CPT-II) confers malonyl-CoA sensitivity to it indicates that this form of CPT may also be the catalytic subunit responsible for malonyl-CoA sensitivity of intact mitochondria. Such a conclusion is consistent with the data of Fritz and colleagues (Kopec & Fritz, 1973), in which it was shown that urea could convert the two CPTs they isolated from bovine mitochondria into a single catalytic component. Other investigations indicate the CPT activity of mitochondria may be due to a single catalytic protein. The studies of Bergstrom and Reitz (1980), Kerner and Bieber (1990), Healy et al. (1988), Fiol et al. (1987), Clarke and Bieber (1981), and Lilly et al. (1992) indicate that mitochondria contain only one catalytic protein capable of transferring medium-chain and long-chain acyl-CoA's to carnitine. Working models have been proposed (Bieber, 1988; Bieber et al., 1992) which suggest that mitochondria contain a single catalytic species of CPT. In addition to the data presented herein, part of the reason for such models is the fact that, to date, a second catalytic CPT associated with mitochondria has not been definitively isolated and identified. Although several reports have suggested the existence of a second catalytic species, the early reports of easily-solubilized mitochondrial CPT must be considered equivocal. To our knowledge, all easily-solubilized CPT activities associated with mitochondria isolated by differential centrifugation, when rigorously characterized, have been shown to be the peroxisomal enzyme that exhibits catalytic activity with both

medium-chain and long-chain acyl-CoAs. In addition, the contribution of the malonyl-CoA-sensitive carnitine palmitoyltransferase activity of microsomes (Lilly et al., 1990; Murthy & Bieber, 1992) to "overt" CPT of liver mitochondria has often been ignored.

For a model to be correct in which mitochondria contain only one catalytic species of CPT, it is essential that the mitochondrial CPT, i.e, CPT_i/CPT-II, be associated with both sides of the inner membrane of mitochondria or be associated with both the outer membrane of mitochondria and the matrix side of the inner membrane of mitochondria. Although the precise location of CPT-I/CPT_o is in debate, our data show malonyl-CoA sensitivity can be conferred to purified CPT-II/CPT_i by addition of proteins that bind malonyl-CoA. The data do not support models which assume the 86-kDa malonyl-CoA binding is a detergent-labile form of CPT or models which assume that CPT-II/CPT_i is incapable of exhibiting malonyl-CoA sensitivity (McGarry et al., 1989); rather, the data indicate mitochondrial CPT can exist in forms that are inhibitable by malonyl-CoA (presence of a regulator subunit) and can exist in forms that are insensitive to malonyl-CoA (nonassociation of a regulator subunit with CPT-II). Alteration of catalytic activity and/or function by association of a catalytic protein with another protein is a well-established phenomenon. A simple example is lactose synthetase, which catalyzes synthesis of *N*-acetyllactosamine in the absence of α-lactalbumin, but in the presence of α-lactalbumin, the acceptor specificity is changed, resulting in lactose synthesis (Voet & Voet, 1990).

REFERENCES

Bennett, M. J. (1990) *Ann. Clin. Biochem. 26*, 1–13.

Bergstrom, J. D. & Reitz, R. C. (1980) *Arch. Biochem. Biophys. 204*, 71–79.

Bieber, L. L. (1988) *Annu. Rev. Biochem. 57*, 261–283.

Bieber, L. L. (1991) in *Current Concepts in Carnitine Research*, pp 7–17, Wiley Press, New York.

Bieber, L. L., Dai, G., & Chung, C. (1991) in *Current Concepts in Carnitine Research*, pp 129–135, Wiley Press, New York.

Brandolin, G., Boulay, F., Dalbon, P., & Vignais, P. V. (1989) *Biochemistry 28*, 1093–1100.

Bremer, J., Woldegiorgis, G., Schalinske, K., & Shrago, E. (1985) *Biochim. Biophys. Acta 833*, 9–16.

Chung, C., Woldegiorgis, G., & Bieber, L. (1991) *FASEB J. 5*, Abstr.

Clarke, P. R. H., & Bieber, L. L. (1981) *J. Biol. Chem. 256*, 9861–9868.

Declercq, P. E., Falck, J. R., Kuwajima, M., Tyminski, H., Foster, D. W., & McGarry, J. D. (1987) *J. Biol. Chem. 262*, 9812–9821.

Fiol. C. J., Kerner, J., & Bieber, L. L. (1987) *Biochim. Biophys. Acta 916*, 482–492.

Ghadiminejad, I., & Saggerson, E. D. (1990a) *FEBS Lett. 269*, 406–408.

Ghadiminejad, I., & Saggerson, E. D. (1990b) *Biochem. J. 270*, 787–794.

Ghadiminejad, I., & Saggerson, E. D. (1991) *Biochim. Biophys. Acta 1083*, 166–172.

Green, D. E., Lester, R. L., & Ziegler, D. M. (1957) *Biochim. Biophys. Acta 23*, 515–524.

Harlow, E., & Lane, D. (1988) in *Antibodies—A Laboratory Manual*, pp 522–523, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY.

Healy, M. J., Kerner, J., & Bieber, L. L. (1988) *Biochem. J. 249*, 231–237.

Hoppel, C. L. (1991) in *Current Concepts in Carnitine Research*, pp 151–163, Wiley Press, New York.

Kashfi, K., & Cook, G. A. (1991) *Biochem. Biophys. Res. Commun. 178*, 600–605.

Kerner, J., & Bieber, L. (1990) *Biochemistry 29*, 4326–4334.

Kolodziej, M. P., & Zammit, V. A. (1991) *Biochem. J. 272*, 421–425.

Kolodziej, M. P., Crilly, P. J., Corstorphine, C. G., & Zammit, V. A. (1992) *Biochem. J. 282*, 415–421.

Kopec, B., & Fritz, I. B. (1973) *J. Biol. Chem. 248*, 4069–4074.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lilly, K., & Bieber, L. (1990) *Life Sci. Adv. 9*, 79–85.

Lilly, K., & Bugaisky, G. E., Umeda, P. K., & Bieber, L. L. (1990) *Arch. Biochem. Biophys. 280*, 167–174.

Lilly, K., Chung, C., Kerner, J., VanRenterghem, R., & Bieber, L. L. (1992) *Biochem. Pharmacol. 43*, 353–361.

Lund, H. (1987) *Biochim. Biophys. Acta 918*, 67–75.

Lund, H., & Woldegiorgis, G. (1987) *Biochim. Biophys. Acta 878*, 243–249.

Markwell, M. A. K., Haas, S. M., Tolbert, N. E., & Bieber, L. L. (1981) *Methods Enzymol. 72D*, 296–303.

McGarry, J. D., Leatherman, G. F., & Foster, D. W. (1978) *J. Biol. Chem. 253*, 4128–4136.

McGarry, J. D., Woeltje, K. F., Kuwajima, M., & Foste, D. (1989) *Diabetes/Metab. Rev. 5*, 271–284.

McGarry, J. D., Sen., A., Weis, B. C., & Foster, D. W. (1991) in *Current Concepts in Carnitine Research*, pp 135–149, Wiley Press, New York.

Murthy, M. S. R., & Pande, S. V. (1987) *Biochem. J. 248*, 727–733.

Murthy, M. S. R., & Pande, S. V. (1990) *Biochem. J. 268*, 599–604.

Murthy, M. S. R., & Bieber, L. L. (1992) in *Current Concepts in Carnitine Research*, Abstr., p 192, Wiley Press, New York.

Murthy, M. S. R., & Pande, S. V. (1991) *Proc. Natl. Acad. Sci. U.S.A. 84*, 378–382.

Murthy, M. S. R., & Bieber, L. L. (1992) *Protein Expression Purif. 3*, 75–79.

Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.

Voet, D., & Voet, J. (1990) in *Biochemistry*, p 570, Wiley Press, New York.

Woldegiorgis, G., Fibich, B., Contreras, L., & Shrago, E. (1989) *FASEB J. 3*, Abstr. 4159, A942.

Woldegiorgis, G., Fibich, B., Contreras, L., & Shrago, E. (1990) *FASEB J. 4*, paper 3106.

Woldegiorgis, G., Fibich, B., Contreras, L., & Shrago, E. (1992) *Arch. Biochem. Biophys. 295*, 348–351.

Registry No. CPT, 9068-41-1; malonyl-CoA, 524-14-1.