Evidence of two catalytically active carnitine medium/long chain acyltransferases in rat liver peroxisomes

Harmeet Singh,[1] Kaye Beckman, and Alfred Poulos

Department of Chemical Pathology, Women's and Children's Hospital, 72 King William Road, North Adelaide, South Australia, 5006, Australia

Abstract Peroxisomal matrix proteins were extracted from the highly purified peroxisomes with sodium pyrophosphate, and the membranes were sedimented by high speed centrifugation. Biochemical marker enzyme analyses revealed a quantitative release of a number of well-known peroxisomal matrix proteins from the purified peroxisomes. In contrast, carnitine medium/long chain acyltransferase activity, assayed with decanoyl-CoA and palmitoyl-CoA as substrates, was equally distributed in the membrane and the matrix fractions. The matrix and the membrane enzyme activities were differentially affected by a number of detergents. The enzyme in the membrane fraction showed higher malonyl-CoA sensitivity compared to the enzyme in the matrix fraction. The enzyme(s) from the purified peroxisomes or the peroxisomal membranes was quantitatively solubilized by sodium cholate, and the cholate-solubilized enzyme retained malonyl-CoA sensitivity. The membrane enzyme was separated from the matrix enzyme by hydroxylapatite column chromatography. The separation of the membrane enzyme or the matrix enzyme by hydroxylapatite column chromatography resulted in loss of malonyl-CoA sensitivity. The partially purified membrane and the matrix enzymes showed broad substrate specificity, and the highest enzyme activities for both were observed with decanoyl-CoA. In contrast to the matrix enzyme, the membrane enzyme was strongly inhibited by high concentrations ($\geq$ 50 μM) of acyl-CoAs (> 10 carbons in length). The matrix enzyme showed a 2.5-fold lower K_m for carnitine compared to the membrane enzyme. The catalytic properties of the partially purified matrix enzyme appear to be similar to the well-characterized peroxisomal carnitine octanoyltransferase, though we find highest activity with decanoyl-CoA rather than octanoyl-CoA as a substrate. The data presented clearly indicate that the membrane and the matrix enzyme activities are due to different proteins.—**Singh, H., K. Beckman, and A. Poulos.** Evidence of two catalytically active carnitine medium/long chain acyltransferases in rat liver peroxisomes. *J. Lipid Res.* 1996. **37:** 2616–2626.

Supplementary key words mitochondria • microsomes • carnitine octanoyltransferase • carnitine palmitoyltransferase • fatty acid transport • fatty acid oxidation

Carnitine medium/long chain acyltransferase or carnitine palmitoyltransferase (CPT, EC 2.3.1.21) activities are present in mitochondria (1). Malonyl-CoA-sensitive enzyme (CPT I) is located in the mitochondrial outer membrane whereas the malonyl-CoA-insensitive enzyme (CPT II) is present in the mitochondrial inner membrane (2, 3). Rat liver as well as human liver CPT II have been isolated and cloned, and the available evidence indicates that the same protein (~71 kDa) is expressed in all body tissues (4–6). In contrast to CPT II, CPT I in rat appears to exist as two isoforms, liver (~88 kDa) and muscle (~82 kDa) enzymes, with different kinetic properties and malonyl-CoA sensitivities (4, 7, 8). Two isoforms of CPT I are expressed in rat heart, the minor component (~88 kDa) is identical to the liver enzyme, and the major component (~82 kDa) is presumably similar to the muscle enzyme (9, 10).

Recent evidence suggests that microsomes contain malonyl-CoA-sensitive and -insensitive carnitine medium/long chain acyltranferases (11). The malonyl-CoA-sensitive enzyme appears to be associated with the microsomal membranes, and a 54 kDa protein, presumably malonyl-CoA-insensitive enzyme, was suggested to be present in the microsomal lumen (11, 12). Carnitine medium/long chain acyltransferase, also known as carnitine octanoyltransferase (COT), has previously been purified from rat and mouse liver peroxisomes (13, 14). The purified COT has been reported to be a malonyl-CoA-insensitive enzyme. Extraction of the enzyme from peroxisomes is thought to result in loss of malonyl-CoA sensitivity (15). We present evidence herein that the peroxisomal malonyl-CoA-sensitive and -insensitive activities are due to two separate proteins. The malonyl-

Abbreviations: CPT, carnitine palmitoyltransferase; COT, carnitine octanoyltransferase; octyl glucoside, n-octyl β-D-glucopyranoside; alkyl-DHAP-synthase, alkyl dihydroxyacetone phosphate synthase; DHAP-AT, dihydroxyacetone phosphate acyltransferase.

[1]To whom correspondence should be addressed.

CoA-sensitive enzyme activity is strongly associated with the peroxisomal membranes compared to the malonyl-CoA-insensitive enzyme, and the enzyme retains malonyl-CoA sensitivity after extraction from the membranes.

MATERIALS AND METHODS

Materials

L-[Methyl-^{3}H]carnitine hydrochloride (82 Ci/mmol) was purchased from Amersham Australia, Sydney, New South Wales. Nycodenz, used for gradients, was obtained from Nycomed Pharma AS, Oslo, Norway. Percoll, dithiothreitol, fatty acid-free BSA, homovanillic acid, peroxidase (type II), glycolic acid, uric acid, D-proline, N′-acetylspermine, sodium cholate, digitonin, CHAPS, Brij-58, n-octyl β-D-glycopyranoside (octyl glucoside), Triton X-100, L-carnitine, malonyl-CoA, and acyl-CoAs were obtained from Sigma Chemical Company, St. Louis, MO. Bio-Gel HT (hydroxylapatite) was purchased from Bio-Rad Laboratories, Richmond, CA. All other reagents and solvents were of analytical grade, and were purchased from May and Baker Australia Pty. Ltd, Melbourne, Victoria, or Ajax Chemicals, Sydney, New South Wales. Adult Wistar rats were supplied by the Agricultural Experiment Station, Gilles Plains, South Australia.

Isolation of rat liver peroxisomes

Adult rats were killed by exposure to CO_2 in gas chambers. Livers were removed and rinsed 3–4 times with 10–15 ml of ice-cold buffered sucrose (0.25 M sucrose–1 mM EDTA–10 mM Tris-HCl buffer, pH 7.5). The livers (35–50g) were finely minced and hand homogenized in 5–6 volumes of the buffered sucrose (above) using a glass homogenizer with a loose-fitting pestle. The resultant homogenate was centrifuged at 500 *g* for 5 min to remove nuclei, debris, and unbroken cells. The supernatant was further centrifuged to obtain crude mitochondrial and crude peroxisomal pellets (16, 17). Peroxisomes were isolated from the crude peroxisomal pellets by Nycodenz and Percoll density gradients, and the purity of the organelles was established (16, 17).

Preparation of peroxisomal membrane and matrix fractions

Purified peroxisomes (10 mg protein/ml) in 20 ml of 5 mM potassium phosphate buffer (pH 7.4) were adjusted to pH 8.5–9.0 with 1 ml of 100 mM tetrasodium pyrophosphate; 200 μl of 100 mM dithiothreitol was added and the suspension was left at 4°C overnight. After 16 h, the suspension was centrifuged at 100,000 *g* for 60 min and the supernatant was removed. The pellet was resuspended in 20 ml of 10 mM tetrasodium pyrophosphate buffer containing 1 mM dithiothreitol (pH 9.0). The suspension was left at 4°C for 2 h and centrifuged as above. The above procedure was repeated on the pellet, and the final pellet (which is referred to throughout as "peroxisomal membranes") was suspended in 20 mM potassium phosphate buffer, pH 7.4. The three supernatant fractions (from above) were combined, and dialyzed overnight (~16 h) against 2 liters of 20 mM potassium phosphate buffer, pH 7.4. Denatured proteins were removed by centrifugation (100,000 *g* for 60 min) and the supernatant obtained was referred to as "peroxisomal matrix."

Fractionation of peroxisomal carnitine medium/long chain acyltransferase by hydroxylapatite (Bio-Gel HT) column chromatography

Rat liver peroxisomes or peroxisomal membranes (5–10 mg protein/ml) were extracted at 4 °C for 2 h with 20 mM potassium phosphate buffer containing 1 mM dithiothreitol and 2% (w/v) sodium cholate (final pH 7.4), and the insoluble proteins were removed by centrifugation (100,000 *g* for 60 min). The cholate-solubilized proteins were loaded (15 ml/h) onto a hydroxylapatite (Bio-Gel HT) column (45 × 1 cm) that was pre-equilibrated with 20 mM potassium phosphate buffer (pH 7.4) containing 1 mM dithiothreitol and 0.2% sodium cholate. The column was washed with 20–50 ml of 20 mM potassium phosphate buffer, pH 7.4, containing 1 mM dithiothreitol and 0.2% sodium cholate. The adsorbed proteins from the column were eluted with a linear gradient (20–400 mM) of potassium phosphate buffer (pH 7.4). A linear gradient was generated using 100 ml each of 20 mM potassium phosphate buffer (pH 7.4) and 400 mM potassium phosphate buffer (pH 7.4), both buffers contained 1 mM dithiothreitol and 0.2% sodium cholate. The proteins from the column were eluted at 8 ml/h, and 8-ml fractions were collected. The peroxisomal matrix proteins were separated on the hydroxylapatite column (Bio-Gel HT) using the same elution conditions as above.

Measurements of carnitine medium/long chain acyltransferase activity

The incubations consisted of 50 mM sodium phosphate buffer, pH 7.0, dithiothreitol (1 mM), L-[methyl-^{3}H]carnitine (20 μCi/μmol, 250 μM), acyl-CoA (100 μM, or as indicated), and enzyme (1–5 μg protein) in a total volume of 0.1 ml. The enzyme assays were performed at 30°C for 5 min (or as indicated), and the reaction was stopped with 2 ml of chloroform–methanol 1:1 (v/v). The two phases were separated by the addition of 0.5 ml of extraction mixture (2 M KCl containing 0.2 M

H_3PO_4). The upper aqueous phase was discarded and the lower chloroform phase was washed once with 0.5 ml of the extraction mixture (as above), and the radioactivity in the chloroform phase (acyl carnitine) was determined. Radiolabeled acyl carnitine produced was quantitatively extracted into the chloroform phase when >10 carbon acyl-CoA (lauroyl-CoA to behenoyl-CoA) was used as a substrate. However, ~75% of the product (decanoyl carnitine) was extracted into the chloroform phase when decanoyl-CoA was used as a substrate. In experiments where octanoyl-CoA was used as a substrate, the incubations were performed as above, but the reaction was terminated by the addition of 1 ml of isobutanol, and the product (octanoyl carnitine) was extracted and quantified as described by Solberg (18). The control experiments, without enzyme, were performed with each set of assays. The control experiments indicate that < 0.1% of radiolabeled carnitine was extracted into the chloroform phase under the above extraction conditions. In contrast, we find that the blank values were at least 20-fold higher with the isobutanol extraction procedure (18).

Other biochemical analyses

Mitochondrial, peroxisomal, microsomal, lysosomal, and plasma membrane marker enzyme activities were assayed as described in earlier publications (16, 17). Catalase, palmitoyl-CoA oxidase, and uric acid oxidase assays were performed as described (16, 17, 19). The assay conditions for polyamine oxidase, D-amino acid oxidase, and glycolate oxidase were the same as described for uric acid oxidase (19). N′-acetylspermine (1 mM), D-proline (5 mM), and glycolic acid (10 mM) were used as the substrates for polyamine oxidase, D-amino acid oxidase, and glycolate oxidase, respectively, and the incubations were performed at 37°C for 30 min (polyamine oxidase and D-amino acid oxidase) or 10 min (glycolate oxidase).

The protein content was determined fluorometrically using human serum albumin as a standard (20). Potassium ion concentrations in the column fractions were determined by flame photometry with 100 mM K^+ and Na^+ ions as standards (Instrumentation Laboratories, Lexington, MA).

RESULTS

The highly purified peroxisomes contained <2% mitochondrial marker enzyme activities namely, α-ketoglutarate dehydrogenase and succinate dehydrogenase (16, 17). The specific activity of carnitine medium/long chain acyltransferase, assayed with decanoyl-CoA as a substrate, was found to be very similar in the purified mitochondria and the peroxisomes. Therefore, we conclude that the contribution of the mitochondrial enzyme in the purified peroxisomal preparations would not exceed 2%. Compared to mitochondria and peroxisomes, the specific activity of carnitine medium/long chain acyltransferase in microsomes was 7- to 8-fold lower (data not given) The specific activities of microsomal marker enzymes (NADPH-cytochrome C reductase, glucose 6-phosphatase, and arylsuphatase C) in the purified peroxisomes were at least 5-fold lower compared to the activities in microsomes (16, 17). Previous studies (21) with purified rat liver peroxisomes clearly indicate that NADPH-cytochrome P-450 reductase and NADH-cytochrome C reductase, the well-known microsomal enzymes, are bona fide peroxisomal proteins. However, even assuming that peroxisomes do not contain any of the microsomal marker enzyme activities and the enzyme activities detected in purified peroxisomal preparations reflect microsomal contamination, we estimate that <3% of the carnitine acyltransferase activity detected in the purified peroxisomes would be due to the microsomal enzyme. Clearly, the degree of microsomal contamination would be much less (possibly <1%) if peroxisomes contained the microsomal enzyme markers. Thus, according to our highly conservative estimates, we find that >95% of the carnitine acyltransferase activity observed in our purified peroxisomal preparations was due to peroxisomes.

Carnitine medium/long chain acyltransferase activity in the purified peroxisomes was investigated with decanoyl-CoA and palmitoyl-CoA as substrates. The enzyme activity was linear with time, at least up to 5 min, and up to 7 μg protein (data not given). A 5-fold higher enzyme activity was observed with decanoyl-CoA compared to palmitoyl-CoA as a substrate (data not given). Without BSA, malonyl-CoA (up to 50 μM) showed only a slight inhibition, whereas in the presence of BSA (100 μM each of fatty acid-free BSA and fatty acyl-CoA) malonyl-CoA strongly inhibited the enzyme activity (**Fig. 1**). Malonyl-CoA inhibition of the enzyme activity depended upon the molar ratio of fatty acid-free BSA and the substrate (fatty acyl-CoA) as well as the chain length of fatty acyl-CoA substrate. A maximum inhibition of the enzyme activity by malonyl-CoA (50 μM) was observed at 2:1 molar ratio of fatty acid-free BSA and palmitoyl-CoA. A higher malonyl-CoA inhibition was observed with palmitoyl-CoA compared with decanoyl-CoA as a substrate for the enzyme (Fig. 1).

Fractionation of peroxisomes by a sodium pyrophosphate extraction procedure resulted in release of 60–65% of the peroxisomal proteins. A quantitative release of a number of peroxisomal matrix enzymes, namely catalase, palmitoyl-CoA oxidase, glycolate oxidase, poly-

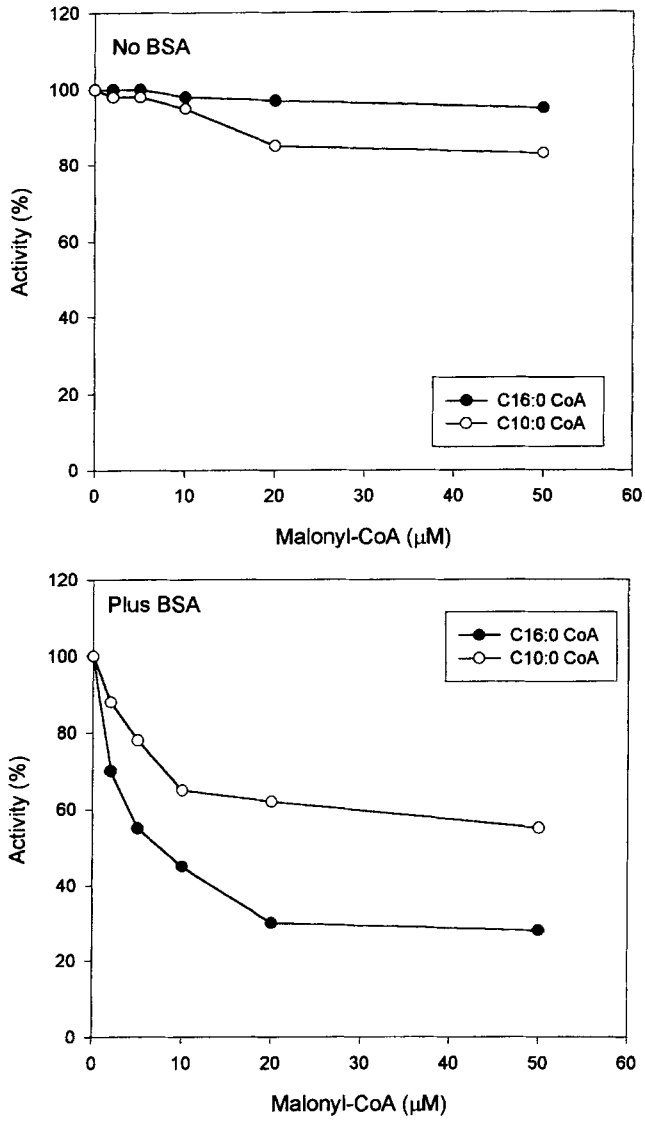

Fig. 1. Malonyl-CoA inhibition of peroxisomal carnitine medium/long chain acyltransferase. The purified rat liver peroxisomes (4–5 μg protein) were preincubated at room temperature for 5 min with indicated concentrations of malonyl-CoA in the incubations (see assay conditions for carnitine medium/long chain acyltransferase) and the enzyme activities were determined with palmitoyl-CoA or decanoyl-CoA as a substrate. The enzyme reaction was initiated by adding substrate (50 μM of acyl-CoA or 50 μM each of acyl-CoA and fatty acid-free BSA), and the incubations were performed at 30°C for 5 min. The radiolabeled product (acyl carnitine) formed during the incubations was separated and quantified (see Methods). The data at each point on the graph are an average of two observations. The experiments were repeated on different preparations of peroxisomes and the results of every preparation examined were similar.

amine oxidase, and D-amino acid oxidase was observed under these conditions (**Table 1**). The peroxisomal membrane marker enzyme, alkyl dihydroxyacetone phosphate synthase (alkyl DHAP-synthase), and the peroxisomal core enzyme, uric acid oxidase, quantitatively sedimented with peroxisomal membranes. Dihydroxyacetone phosphate acyltransferase (DHAP-AT), another peroxisomal membrane enzyme, activity was detected in both the matrix and the membrane fractions suggesting that some of the membrane proteins were also extracted by sodium pyrophosphate. Carnitine medium/long chain acyltransferase activity assayed with decanoyl-CoA as a substrate showed an equal distribution in the membrane and the matrix fractions (Table 1). A similar distribution of the enzyme activity was observed with palmitoyl-CoA as a substrate, but the specific activities were 4- to 5-fold lower.

The effect of increasing concentrations of a number of detergents on the peroxisomal membrane and the matrix carnitine medium/long chain acyltransferase activities was investigated. Low concentrations (up to 0.1%) of sodium cholate stimulated the membrane enzyme activity, and the higher concentrations (>0.1%) were slightly inhibitory (**Fig. 2C**). In contrast, the matrix enzyme activity was inhibited by sodium cholate (Fig. 2A). Octyl glucoside at concentrations up to 0.2% stimulated the membrane enzyme activity, but it had no effect on the matrix enzyme activity (Fig. 2B,D). Brij-58, digitonin and Triton X-100 strongly inhibited the membrane enzyme activity compared to the matrix enzyme activity (Fig. 2). CHAPS inhibited both the membrane and the matrix enzyme activities, but the membrane activity was more strongly inhibited compared to the matrix activity at 0.15% and 0.2% CHAPS (Fig. 2A,C).

Carnitine medium/long chain acyltransferase activity was quantitatively (>95%) solubilized from peroxisomes or peroxisomal membranes by 2% (w/v) sodium cholate. Solubilization of the enzyme from the purified peroxisomes or the peroxisomal membranes by sodium cholate resulted in retention of malonyl-CoA sensitivity (**Fig. 3**). As expected, higher malonyl-CoA inhibition of the enzyme was observed with palmitoyl-CoA compared with decanoyl-CoA as a substrate. Relatively stronger inhibition by malonyl-CoA was observed at higher molar ratio (2:1) of fatty acid-free BSA and fatty acyl-CoA (compare Figs. 1 and 3). Solubilization of the enzyme from peroxisomes or peroxisomal membranes by sodium cholate resulted in decreased malonyl-CoA sensitivity irrespective of whether palmitoyl-CoA or decanoyl-CoA was used as a substrate (Fig. 3). The release of the enzyme from the purified peroxisomes without the aid of a detergent (for example, extraction of the enzyme from the purified peroxisomes by pyrophosphate) also resulted in decreased malonyl-CoA sensitivity (data not given).

Fractionation of the cholate-solubilized proteins from peroxisomes on hydroxylapatite columns gave two major peaks of the enzyme activities. A sharp peak eluting with 50 mM potassium phosphate and a broad peak

TABLE 1. Enzyme activities in purified peroxisomes, the peroxisomal matrix, and the membrane fractions

	Specific Activity			Total Activity	
	Peroxisomes	Matrix	Membranes	Matrix	Membranes
				%	
Catalase[a]	12.4	18.3	0.1	99	1
Palmitoyl-CoA oxidase[b]	44.5	93.4	1.7	98	2
Glycolate oxidase[b]	67.9	110.2	0	100	0
Polyamine oxidase[b]	2.1	4.3	0	100	0
D-Amino acid oxidase[b]	17.2	28.2	0.3	99	1
Uric acid oxidase[b]	176.2	0	390.2	0	100
Akyl-DHAP-synthase[c]	29.3	0.6	72.7	1	99
DHAP-AT[b]	31.7	10.5	38.2	15	85
Carnitine decanoyl transferase[b]	10.4	19.3	12.7	51	49

Highly purified rat liver peroxisomes were subjected to sodium pyrophosphate treatment and the matrix and the membrane fractions were isolated as described in Methods. The biochemical assays were performed in duplicate on peroxisomes and peroxisomal fractions (see Methods) and the data are presented as an average of two observations. The recoveries of the enzyme activities ranged from 88 to 100% and the protein recoveries were always >85%. The enzyme activities were assayed in three separate preparations and the range of values obtained was within 5%.

[a]mmol/min/mg protein.
[b]nmol/min/mg protein.
[c]nmol/h/mg protein.

eluting with 220 mM potassium phosphate (**Fig. 4A**). Separation of pyrophosphate-extracted peroxisomal proteins (peroxisomal matrix) on hydroxylapatite columns resulted in a major peak of the enzyme eluting with 50 mM potassium phosphate and a minor peak of the enzyme eluting with 220 mM potassium phosphate (Fig. 4B). However, the minor peak was not detected when sodium cholate was omitted from the gradient buffers (data not given). Fractionation of the cholate-solubilized peroxisomal membrane proteins on hydroxylapatite column gave only one peak of the enzyme activity eluting with 220–250 mM potassium phosphate (Fig. 4C). The enzyme activity could not be eluted from the column when sodium cholate was omitted from the gradient buffers (data not given). As expected, the protein profile from hydroxylapatite columns was clearly different with the peroxisomal membrane and the matrix fractions (Fig. 4B and 4C).

The rates of reaction with the partially purified matrix and the membrane enzymes (peak 1 of Fig. 4B and peak fraction of Fig. 4C) were linear with incubation period at least up to 5 min with decanoyl-CoA, and up to 15 min with arachidoyl-CoA and behenoyl-CoA as substrates (data not given). Also, the partially purified membrane and the matrix enzyme activities were linear at least up to 5 μg protein with decanoyl-CoA as a substrate (data not given).

Substrate saturation studies with the partially purified matrix enzyme indicated that the enzyme can utilize a range of acyl-CoAs as substrates. The activity increased with the increasing substrate concentration, and the activity plateaued at >100 μM (**Fig. 5**). The highest enzyme activity was detected with decanoyl-CoA followed by octanoyl-CoA. The activities decreased with the increasing chain length of fatty acyl-CoA substrate (**Table 2,** Fig. 5). Stearoyl-CoA, arachidoyl-CoA, and behenoyl-CoA were readily converted to their corresponding carnitine esters but the rate of reaction dropped dramatically with acyl-CoAs of ≥18 carbons. We also examined the substrate specificity of the enzyme in the presence of sodium cholate in the incubation assays to exclude the possibility of relative insolubility and/or nonspecific binding of the longer chain acyl-CoAs to the proteins. The substrate specificity of the enzyme was unaltered by the inclusion of sodium cholate (0.1%, w/v, final concentration) in the incubations. With decanoyl-CoA as a substrate, the apparent K_m of the enzyme for L-carnitine was estimated to be 125 μM.

The partially purified peroxisomal membrane enzyme also showed broad substrate specificity. The rate of reaction increased with increasing concentrations of acyl-CoAs. The highest enzyme activity was observed at 20 μM or 50 μM, depending upon the acyl-CoA used (**Fig. 6**). Octanoyl-CoA up to 200 μM did not inhibit the enzyme reaction. Decanoyl-CoA at concentration > 50 μM was slightly inhibitory but much stronger inhibition of the enzyme activity was observed with acyl-CoAs of chain length > 10 carbons (Fig. 6). For example, myristoyl-CoA, palmitoyl-CoA, and stearoyl-CoA showed strong inhibition of the enzyme activity at concentrations > 20 μM. Inclusion of 50–100 μM fatty acid-free BSA in the incubations prevented substrate inhibition of the enzyme (data not given). The partially purified peroxisomal membrane enzyme showed highest activity with decanoyl-CoA followed by lauroyl-CoA and myristoyl-CoA (Table 2). The activity with palmitoyl-CoA was

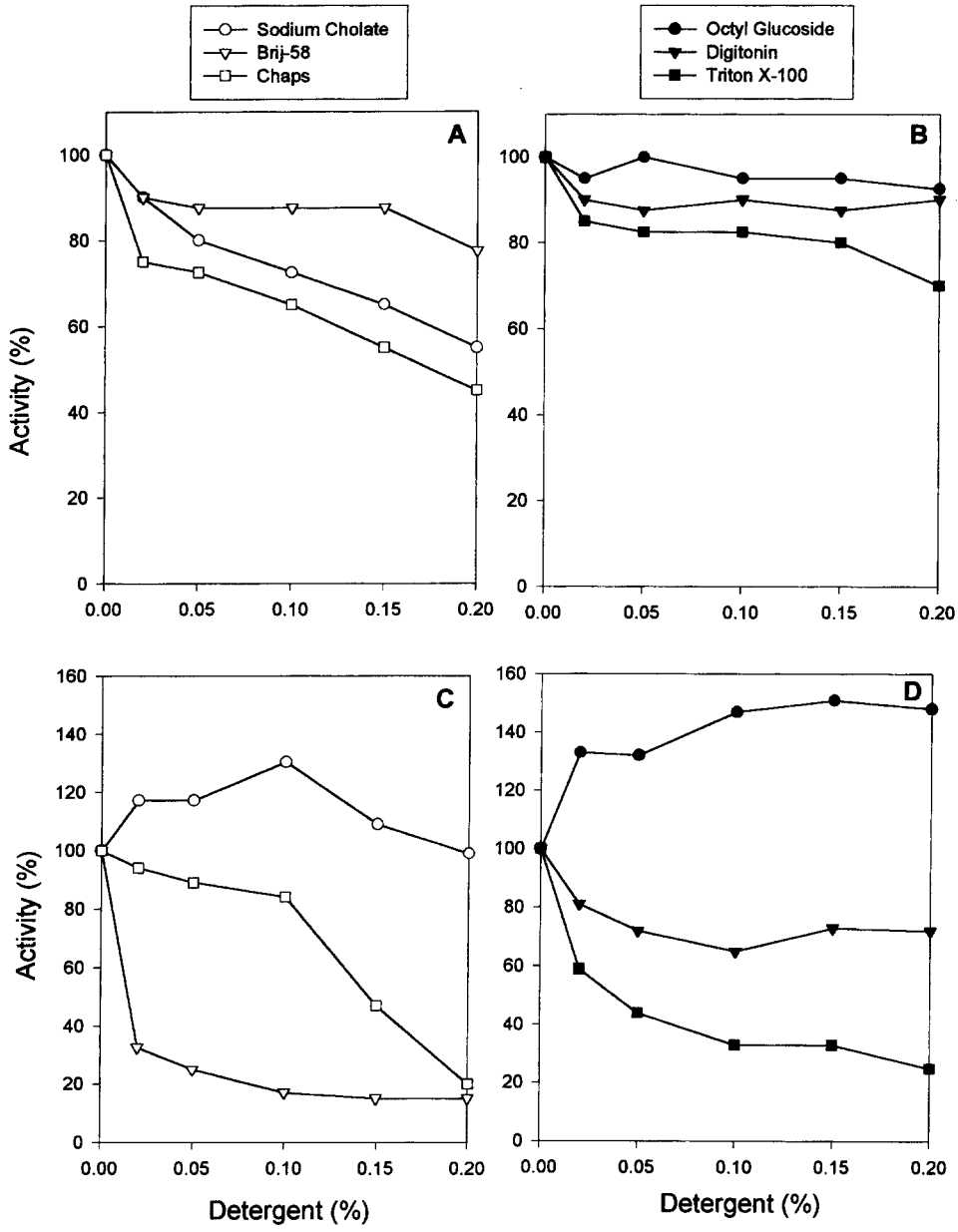

Fig. 2. The effect of detergents on the peroxisomal membrane and the matrix enzyme activities. Carnitine acyltransferase activity in the matrix fraction (A,B) or the membrane fraction (C,D) was assayed at 30°C for 5 min with 3–4 μg protein in the presence of indicated concentrations of the detergent in the incubations. Fifty μM and 100 μM of unlabeled decanoyl-CoA was used as the substrate for the assay of the membrane and the matrix enzyme activities, respectively. The amount of radiolabeled decanoyl carnitine formed during the incubations was determined (see Methods), and the data are presented as an average of two observations. The experiments were repeated on a different preparation and similar results were obtained.

4- to 5-fold lower compared to decanoyl-CoA, and the activity with stearoyl-CoA and arachidoyl-CoA was very low (Fig. 6, Table 2). The substrate specificity of the enzyme was unaffected in the presence of sodium cholate (0.1% w/v, final concentration) in the incubations. With decanoyl-CoA as a substrate, the apparent K_m for the enzyme for L-carnitine was estimated to be 330 μM.

DISCUSSION

The sodium pyrophosphate extraction procedure used quantitatively extracted a number of known matrix proteins from the purified peroxisomes without inactivating the enzyme activities associated with those proteins. The method described clearly separates the mem-

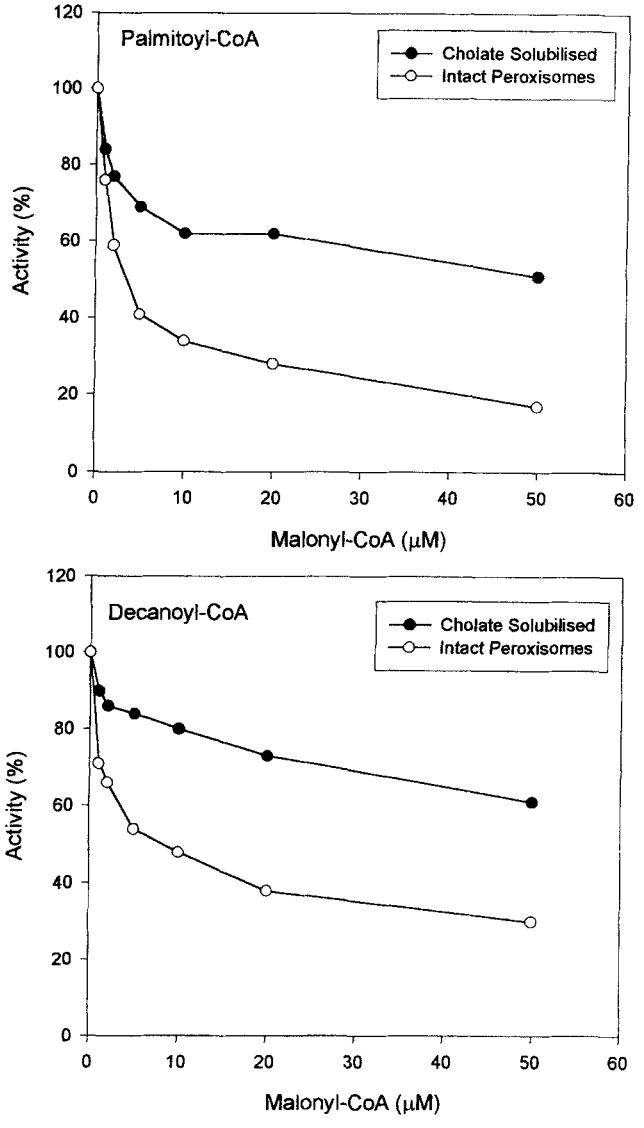

Fig. 3. Malonyl-CoA inhibition of the peroxisomal enzyme(s) before and after solubilization by sodium cholate. The malonyl-CoA inhibition of the enzyme in the purified peroxisomes was determined before and after solubilization of the enzyme with 2% sodium cholate (see Methods) Peroxisomal proteins before and after solubilization (3–5 μg protein) were preincubated for 5 min at room temperature with the indicated concentrations of malonyl-CoA. The enzyme reaction was initiated by the addition of 100 μM of palmitoyl-CoA or decanoyl-CoA and 200 μM of fatty acid-free BSA. The final concentration of sodium cholate in the incubations with sodium cholate-solubilized peroxisomes was 0.02%. The incubations were performed at 30°C for 5 min and the radiolabeled product (acyl carnitine) formed during the incubations was determined (see Methods). Each point on the graph represents an average of two observations. Malonyl-CoA inhibition experiments were repeated using a different preparation and similar results were obtained.

brane proteins from the matrix proteins, though partial extraction of one of the peroxisomal membrane enzymes, DHAP-AT, was also observed (Table 1). Another peroxisomal membrane enzyme, namely long chain acyl-CoA synthetase, was inactivated by sodium pyrophosphate extraction of peroxisomes. The core protein (uric acid oxidase) was not extracted by sodium pyrophosphate (Table 1). In contrast to the known peroxisomal matrix enzymes, carnitine medium/long chain acyltransferase activity was only partially extracted (Table 1), suggesting the possibility of two enzymes in peroxisomes, a membrane-associated enzyme and a matrix enzyme. We observed an equal distribution of carnitine medium/long chain acyltransferase activity in the peroxisomal membrane and the matrix fractions. In an earlier study 30–50% of the enzyme activity was reported to appear in the high speed supernatant upon sonication of purified rat liver peroxisomes (15). In contrast, a preliminary report presented at a conference proceeding (22) indicated that <10% of the enzyme activity was released by limited sonication of peroxisomes. The different results obtained by sonication of peroxisomes may relate to the sonication conditions or the purity of the organelle used.

The differential effect of a number of detergents on the peroxisomal membrane and the matrix enzyme activities further suggest the presence of two enzymes in peroxisomes. The previous reports as well our current observations indicate that the peroxisomal membrane-associated enzyme activity is strongly inhibited by malonyl-CoA compared to the enzyme readily released by gentle sonication (15, 22) or pyrophosphate extraction (current studies) of peroxisomes. The current and the previous observations on malonyl-CoA inhibition may relate to the membrane environment of the enzyme, and the release of the enzyme from the native membrane without detergent, for example sonication or pyrophosphate treatment, results in decreased malonyl-CoA sensitivity. However, the differential effect of a number of detergents on the activity of the membrane and the matrix enzymes cannot be explained by differences in the micro-environment of the same enzyme protein in two different fractions.

The observation that fractionation of peroxisomal proteins by hydroxylapatite column chromatography gave two distinct peaks of the enzyme activities further indicates that peroxisomes contain two distinct carnitine acyltransferases. Also, the peroxisomal membrane-associated enzyme was strongly inhibited whereas the peroxisomal matrix enzyme was not inhibited by high concentrations of long chain acyl-CoAs (>10 carbons). Furthermore, the matrix enzyme was quantitatively released whereas the membrane enzyme was partially released from the purified peroxisomes with sodium pyro-

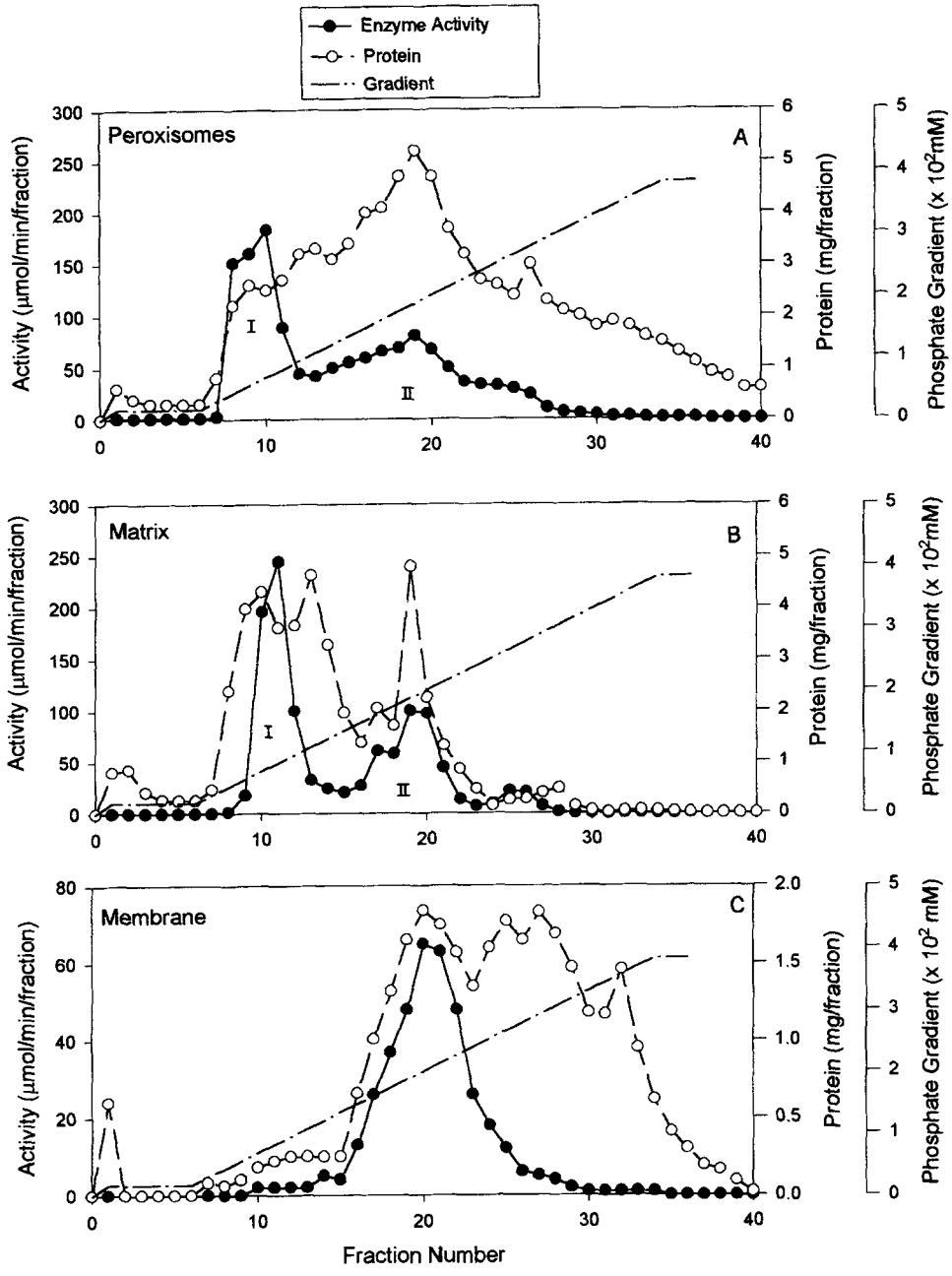

Fig. 4. Separation of carnitine acyltransferases by hydroxylapatite column chromatography. The purified peroxisomes or the peroxisomal membrane proteins were solubilized with sodium cholate (2%, w/v) and loaded (15 ml/h) onto a hydroxylapatite (Bio-Gel HT) column (45 × 1 cm). Sodium cholate (0.2%, final concentration) was also added to the pyrophosphate-solubilized matrix fraction and loaded onto a hydroxylapatite column as above. Unadsorbed proteins were removed by washing the column with 20–50 ml of buffer (20 mM potassium phosphate buffer, pH 7.4, containing 1 mM dithiothreitol and 0.2% sodium cholate). The adsorbed proteins from the column were eluted overnight with a linear gradient of potassium phosphate (see Methods). Fractions (8 ml/h) were collected and analyzed for enzyme activity, protein content, and phosphate concentration (see Methods). The enzyme activity measurements were performed at 30°C for 5 min with 100 µM decanoyl-CoA as a substrate. The fractionation experiments were repeated and the elution profiles of the enzyme and proteins were found to be essentially the same.

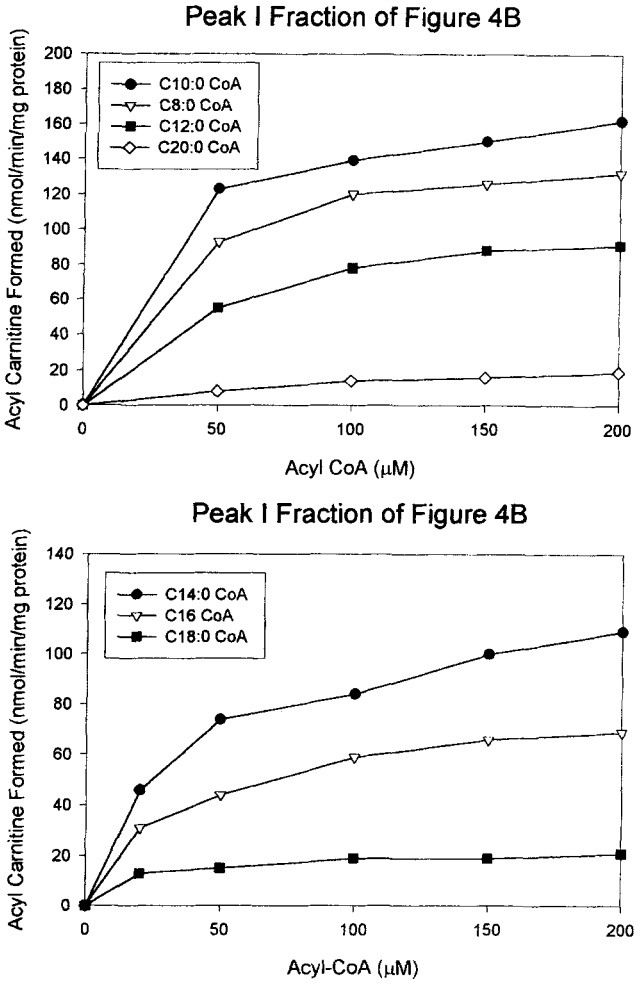

Fig. 5. Substrate saturation studies with the partially purified peroxisomal matrix enzyme. The partially purified matrix (peak I of Fig. 4B) enzyme (4–5 μg protein) was incubated at 30°C for 5 min with the indicated concentrations of unlabeled acyl-CoA and 250 μM of radiolabeled L-carnitine The radiolabeled acyl carnitine formed during the incubations was determined. Each point on the graph represents an average of two observations, and the range of values in an experiment was within 2%. The experiments were repeated and similar results were obtained with a different preparation.

TABLE 2. Substrate specificity of the partially purified peroxisomal matrix and the membrane enzymes

	Matrix Enzyme Substrate Concentration		Membrane Enzyme Substrate Concentration	
Substrate	20 μM	50 μM	20 μM	50 μM
	%		%	
Decanoyl-CoA	100	100	100	100
Octanoyl-CoA	93	93	37	53
Lauroyl-CoA	62	67	85	65
Myristoyl-CoA	42	43	67	35
Palmitoyl-CoA	29	34	22	6
Stearoyl-CoA	13	15	7	3

The peroxisomal matrix enzyme (peak I fraction of Fig. 4B) or the membrane enzyme (peak fraction of Fig. 4C) activities were measured with 250 μM L-carnitine and the indicated concentration of acyl-CoA (see Methods). The enzyme assays were performed in duplicate with 4–5 μg protein at 30°C for 2 min (20 μM acyl-CoA) or 5 min (50 μM acyl-CoA). The radiolabeled product (acyl carnitine) formed during the incubations was separated from the radiolabeled substrate (L-carnitine) and quantified (see Methods). The data are presented as the relative activities with the indicated substrate compared to decanoyl-CoA. The experiments were repeated with a different preparation and similar results were obtained.

phosphate (compare Fig. 4B with 4C). We postulate that the enzyme activity peak I (Fig. 4A and 4B) is a peroxisomal carnitine octanoyl transferase (COT), an enzyme that has been solubilized and purified from rat and mouse liver without the use of detergent (13, 14). We also fractionated peroxisomal matrix proteins on hydroxylapatite columns and used the same conditions (described for Fig. 4), but omitted detergent (sodium cholate) from the loading and elution buffers. Under these conditions, only one peak of enzyme activity (a similar elution profile as peak I of Fig. 4A or 4B) was eluted from the column. The activity was found to be malonyl-CoA-insensitive. These observations further suggest that the enzyme activity in peak I (Fig. 4A or 4B) is due to COT. The enzyme activity in peak II (Fig. 4B) could not be eluted from hydroxylapatite columns unless detergent (sodium cholate) was present in the elution gradients, suggesting further that this enzyme activity is different from COT. We believe that the peak II activity (Fig. 4B) is not an artifact produced by pyrophosphate extraction of peroxisomes for the following reason. We subjected the peak I enzyme (Fig. 4B) to pyrophosphate (as described under Methods for extraction of peroxisomal enzymes by pyrophosphate) and rechromatographed the enzyme on another hydroxylapatite column. We did not find any change in the elution profile of the enzyme, and the enzyme eluted from the column as a single peak. It is interesting to note that at least one of the peroxisomal membrane enzymes, DHAP-AT, was also partially released from the peroxisomal membranes by pyrophosphate (Table 1). The similarities in the elution profile for the matrix enzyme peak II (Fig. 4B) and the membrane enzyme activity (Fig. 4C) indicate that this activity (peak II of Fig. 4B) is due to a peroxisomal membrane enzyme. We speculate that peak II is the malonyl-CoA-sensitive enzyme, some of which is released from the peroxisomal membranes by pyrophosphate. The malonyl-CoA-sensitive enzyme activity observed in the pyrophosphate extracts of intact peroxisomes (data not given) is also possibly due to peak II enzyme (Fig. 4B). Solubilization of the membrane enzyme by sodium cholate results in decreased malonyl-CoA sensitivity as observed with the purified peroxisomes, possibly due to partial dissociation

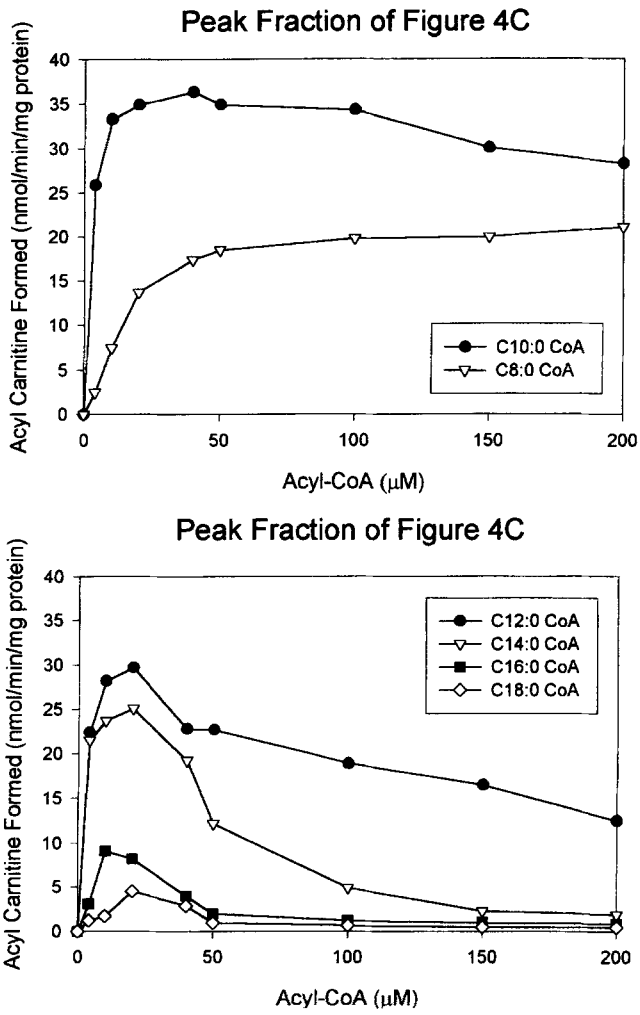

Fig. 6. Substrate saturation studies with the partially purified peroxisomal membrane enzyme. The partially purified membrane (peak fraction of Fig. 4C) enzyme (4–5 μg protein) was incubated at 30°C for 2 or 5 min with indicated concentrations of unlabeled acyl-CoA and 250 μM radiolabeled L-carnitine. The incubation period was 2 min where <50 μM concentrations of acyl-CoA were used as the substrate. The radiolabeled substrate was separated from the product (acyl carnitine) as described under Methods. Each point on the graph represents an average of two observations where the range of values within the experiment was <2%. The substrate saturation experiments were repeated with a different preparation with very similar results.

of the membrane phospholipids or a malonyl-CoA binding protein from the catalytically active protein. Alternatively, sodium cholate alters the configuration of the enzyme resulting in decreased malonyl-CoA binding. Partial purification of the peroxisomal membrane enzyme by hydroxylapatite column chromatography resulted in loss of malonyl-CoA sensitivity possibly due to complete separation of membrane phospholipids or malonyl-CoA binding protein from the catalytically active enzyme. To the best of our knowledge this is the first report to date providing direct evidence of the presence of two catalytically active enzyme activities in purified peroxisomes.

Studies described herein do not exclude the possibility that the peroxisomal COT in the intact organelle is a malonyl-CoA-sensitive enzyme as reported (15), and the release of the enzyme from the organelle either by sonication (15) or by pyrophosphate treatment (present studies) results in reduction of malonyl-CoA sensitivity possibly due to partial dissociation of phospholipids or the malonyl-CoA binding protein from the catalytically active enzyme. The report (23) that the purified COT can be inhibited by malonyl-CoA is surprising in view of our observations that the partially purified COT, the enzyme purified from peroxisomes without the use of detergent, was not inhibited by malonyl-CoA.

The results presented herein provide evidence that peroxisomes, like mitochondria and microsomes, contain malonyl-CoA-sensitive and -insensitive carnitine medium/long chain acyltransferases. The malonyl-CoA-sensitive peroxisomal membrane enzyme is different from the malonyl-CoA-sensitive mitochondrial CPT I for the following reasons. The peroxisomal membrane enzyme can be solubilized from the peroxisomal membranes by sodium cholate and the cholate-solubilized enzyme retains malonyl-CoA sensitivity. In contrast, solubilization of the mitochondrial CPT I from the mitochondrial outer membranes by sodium cholate or sodium deoxycholate results in loss of enzyme activity and malonyl-CoA sensitivity (data not given; see also ref. 24). The peroxisomal membrane enzyme can be partially released from the membranes by pyrophosphate whereas the mitochondrial CPT I cannot be released from the mitochondrial outer membranes by pyrophosphate (data not given). The malonyl-CoA-sensitive enzyme reported in microsomal membranes (11, 24) differs from the malonyl-CoA-sensitive enzyme in the peroxisomal membranes in the following ways. First, the apparent K_m of the peroxisomal membrane enzyme for decanoyl-CoA was estimated to be 7.7 μM compared to 1.1 μM reported for the microsomal membrane enzyme (24). Second, the peroxisomal membrane enzyme showed at least 2-fold higher activity (Table 2) whereas the microsomal membrane enzyme showed 2-fold lower activity with decanoyl-CoA compared to octanoyl-CoA as a substrate (24). The role of malonyl-CoA-sensitive and -insensitive enzymes in mitochondria is well established. In contrast, the role of malonyl-CoA-sensitive and -insensitive enzymes in peroxisomes and microsomes is unclear at present. Further studies on the peroxisomal and microsomal enzymes will provide clues to their function in the cell.

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