Substrate specificity of rat liver mitochondrial carnitine palmitoyl transferase I: evidence against α-oxidation of phytanic acid in rat liver mitochondria

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Abstract The two branched chain fatty acids pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) and phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) were converted to coenzyme A thioesters by rat liver mitochondrial outer membranes. However, these branched chain fatty acids could not be converted to pristanoyl and phytanoyl carnitines, respectively, by mitochondrial outer membranes. As expected, the unbranched long chain fatty acids, stearic acid and palmitic acid, were rapidly converted to stearoyl and palmitoyl carnitines, respectively, by mitochondrial outer membranes. These observations indicate that the branched chain fatty acids could not be transported into mitochondria. The data presented strongly suggest that in rat liver, α-oxidation of phytanic acid occurs in organelles other than mitochondria.

Key words: Fatty acid; Phytanic acid; Pristanic acid; Peroxisome; Mitochondrion; Transport

1. Introduction

Transport of unesterified long chain fatty acids across mitochondrial membranes is a complex biochemical process which involves the actions of long chain acyl-CoA synthetase, carnitine palmitoyl transferase (CPT) I and II, and possibly carnitine/acyl carnitine translocase. Long chain acyl-CoA synthetase, which activates fatty acids to fatty acyl-CoA, is located on the outer (cytoplasmic) surface of mitochondrial outer membranes. The catalytic site of CPTI has been shown to be located on the inner surface of mitochondrial outer membranes [1,2]. Acyl carnitines generated by the action of CPTI are translocated across the mitochondrial inner membrane by carnitine/acyl carnitine translocase [3]. The catalytic site of CPTII is on the inner (matrix) surface of the mitochondrial inner membrane (reviewed by Bieber [4]).

CPTI has recently been purified from rat liver mitochondria and shown to be distinct from CPTII [5]. Apart from mitochondrial CPTI and II, carnitine acyl transferases have also been detected in microsomes, peroxisomes and plasma membranes [6–10]. The carnitine acyl transferase or carnitine octanoyl transferase (COT) purified from rat, mouse and beef liver is a peroxisomal matrix enzyme [6,7,9,11]. Microsomal carnitine acyl transferase is not inhibitied by COT or CPTII antibodies, indicating that it is a distinct protein [8]. McGarry et al. [12] first demonstrated that CPTI activity is reversibly inhibited by malonyl-CoA. Recently, it has been shown that malonyl-CoA inhibits peroxisomal, microsomal and plasma membrane carnitine acyl transferases [8,10,13]. Therefore, in order to understand the role of CPTI in fatty acid oxidation, CPTI must be separated from other carnitine acyl transferases. In view of the difficulties in isolating CPTI in the active state, we decided to investigate CPTI activity in highly purified mitochondrial outer membranes from rat liver. We present evidence that branched chain fatty acids with α- or β-methyl groups are poor substrates for CPTI, suggesting that these branched chain fatty acids cannot be transported into mitochondria via the carnitine/acylcarnitine transport system.

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Abbreviations: CPT, carnitine palmitoyl transferase or carnitine acyltransferase; COT, carnitine octanoyl transferase; TLC, thin layer chromatography.

2. Materials and methods

2.1. Isolation of mitochondria and mitochondrial outer membranes from rat liver

Liver mitochondria were prepared from adult rats (Porton strain) and the purity of the organelles was established [14]. Mitochondrial outer membrane fractions were isolated from purified mitochondria according to the method of Bhuiyan and Pande [15].

2.2. Synthesis of [1-^{14}C]fatty acids and [1-^{14}C]fatty acyl-CoA

[1-^{14}C]Phytanic acid (47 mCi/mmol) was synthesized according to the method of Poulos et al. [16]. [1-^{14}C]Pristanic acid (47 mCi/mmol) was synthesized as described previously [17]. The radiolabelled fatty acids were purified by thin layer chromatography (TLC) and the structures were confirmed by gas chromatography mass spectrometry [18]. The radiolabelled purity of fatty acids was >99%. Radiolabelled fatty acids were converted to fatty acyl chloride by reacting with oxalyl chloride, and coupled with coenzyme A [19]. The radiolabelled acyl-CoA was purified by TLC on silica gel 60 plates using butanol/water/acetic acid (60:20:7, by volume) as the solvent. Radiolabelled acyl-CoA was visualised by autoradiography, scraped and eluted from silica gels with chloroform/methanol/water (50:50:10, by volume). Unlabelled phytanoyl-CoA was synthesized according to the above procedure. Unlabelled stearoyl-CoA and palmitoyl-CoA were purchased from Sigma Chemical Co., St. Louis, MO, USA.

2.3. Fatty acyl-CoA synthetase

Radiolabelled fatty acids were dispersed in α-cyclodextrin solution (20 mM) by sonication [20], and used for acyl-CoA synthetase assay. The assay conditions were the same as described for carnitine acyl transferase (method 1, see section 2.4.1), except L-carnitine was omitted from the incubations. Radiolabelled acyl-CoA produced from [1-^{14}C]fatty acid was measured as described previously [21].

2.4. Carnitine acyl transferase

Carnitine acyl transferase activity was assayed by two different procedures.

2.4.1. Method 1. The incubations consisted of 50 mM Tris-HCl buffer (pH 8.0), ATP (4 mM), Mg^{2+} (4 mM), coenzyme A (0.5 mM), dithiothreitol (1 mM) and L-carnitine (2 mM) in a total volume of 0.2 ml. Radiolabelled fatty acids or fatty acyl-CoA (5–15 μM) dispersed in α-cyclodextrin (2 mM) were added and incubated at 37°C with

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indicated amounts of mitochondrial outer membrane proteins. The reaction was terminated after 5 min with 4 ml of chloroform/methanol (2:1, v/v). The solvent was evaporated under a stream of nitrogen and the extract was dissolved in methanol/water (80:20, v/v). In incubations where radiolabelled fatty acid was used as a substrate, the unreacted substrate was removed by extraction with hexane. Radiolabelled fatty acyl-CoA was separated from fatty acyl carnitine by DEAE-Sephacyl column (6 × 80 mm) chromatography [22]. Alternatively, after evaporation of the solvent, the reaction product was chromatographed on TLC silica gel 60 plates with butanol/water/acetic acid (60:20:7, by volume) as the solvent. After chromatography, TLC plates were subjected to autoradiography, the acyl carnitine region from silica gel plates was scraped, and the radioactivity in acyl carnitine was determined. Negligible acyl carnitine production was observed when L-carnitine was omitted from the incubations. Control experiments without L-carnitine were performed with all the substrates investigated.

2.4.2. Method II. For kinetic experiments, radiolabelled carnitine and unlabelled fatty acyl-CoAs were employed. ATP, Mg^{2+} and coenzyme A were omitted from the incubations. Fatty acid-free bovine serum albumin was included in the incubations to prevent inactivation of CPTI due to the detergent effects of fatty acyl-CoAs. The incubations consisted of 50 mM sodium phosphate buffer, pH 7.0, dithiothreitol (1 mM), L-[methyl-^{3}H]carnitine (40 μCi/μmol, 250 μM), fatty acid-free bovine serum albumin (100 μM) and fatty acyl-CoAs (100 μM) in a total volume of 0.1 ml. Incubations were performed at 30°C for 5 min with mitochondrial outer membranes (1–8 μg protein). The reaction was terminated with 2 ml of chloroform/methanol (1:1, v/v). Two phases were obtained after the addition of 0.5 ml of extraction mixture (2 M KCl containing 0.2 M H_3PO_4). The upper aqueous phase containing radiolabelled carnitine was discarded and the chloroform phase was washed once with a 0.5 ml of the extraction mixture (above). Radiolabelled product, acyl carnitine, formed during the incubations was extracted into the chloroform phase and was quantified. We find that acyl carnitines with acyl moieties >10 carbons in length were quantitatively extracted in the chloroform phase. Control experiments without mitochondrial outer membranes were performed with each set of assays. Control experiments indicated that <0.1% of radiolabelled substrate (carnitine) was extracted into the chloroform phase under the above extraction conditions.

2.5. Monoamine oxidase

Tyramine was employed as the substrate for monoamine oxidase, and substrate-dependent H_2O_2 production was measured fluorometrically. The detection method was based upon the conversion of non-fluoroscent homovanillic acid to a highly fluorescent dimer in the presence of free radicals [23]. The incubations consisted of 50 mM Tris-HCl buffer, pH 8.0, tyramine (1 mM), Triton X-100 (0.1%), horseradish peroxidase (40 μg/incubation) and homovanillic acid (80 μg/incubation) in a total volume of 0.2 ml. Incubations were performed with rat liver factions (3–30 μg protein) at 37°C for 30 min. The reaction was terminated with 1 ml of 50 mM KOH, and the fluorescent product formed during the incubations was quantified fluorometrically (excitation 310 nm, emission 420 nm). Control incubations without the enzyme and also without substrate were included with each liver fraction.

2.6. Other marker enzymes

The assay conditions for uric acid oxidase were similar to those described above for monoamine oxidase. Uric acid oxidase activity was determined using 50 mM sodium phosphate buffer, pH 8.0, and 1 mM uric acid as a substrate. Incubations were performed at 37°C for 15 min using 25–100 μg of liver fractions as an enzyme source. Acyl-CoA oxidase assays were performed at 37°C for 10 min using palmitoyl-CoA (0.25 mM) as a substrate [24]. The assay conditions for dihydroxyacetone phosphate acyltransferase and α-ketoglutarate dehydrogenase were essentially the same as described previously [14,24]. Protein content in liver fractions was determined fluorometrically [25] using human serum albumin as a standard.

3. Results

It is evident from Table 1 that purified mitochondria was enriched in monoamine oxidase (mitochondrial outer membrane enzyme) and α-ketoglutarate dehydrogenase (mitochondrial matrix enzyme) activities. There was at least 15-fold enrichment of monoamine oxidase activity in mitochondrial outer membranes compared to purified mitochondria (Table 1). Only small amounts of α-ketoglutarate dehydrogenase activity was detected in purified mitochondrial outer membranes. Succinate dehydrogenase (a mitochondrial inner membrane enzyme) activity could not be detected in purified outer membrane preparations (data not given), suggesting further that mitochondrial outer membrane preparations contain negligible (<3%) mitochondrial contamination. Plasma membrane enzyme (5′-nucleotidase) activity could not be detected either in purified mitochondria or mitochondrial outer membranes, indicating that these preparations were free of plasma membranes. Negligible activity of dihydroxyacetone phosphate acyltransferase (a peroxisomal membrane enzyme) was detected in purified mitochondria and mitochondrial outer membranes compared to purified peroxisomes (Table 1). Also, acyl-CoA oxidase (a peroxismal matrix enzyme) and uric acid oxidase (a peroxisomal core enzyme) activities were negligible in purified mitochondria, and these enzyme activities could not be detected in purified mitochondrial outer membrane preparations (Table 1). Thus, it is evident that highly purified mitochondria and mitochondrial outer membrane preparations were employed for investigations of acyl-CoA synthetase and carnitine acyltransferase activities.

Acyl-CoA synthetase activity in purified mitochondria was linear with protein up to 2 μg and incubation period up to 5 min using stearic acid as a substrate. Comparable activities of acyl-CoA synthetase were detected in purified mitochondria using palmitic and stearic acids as substrates. The branched chain fatty acids, namely pristanic and phytanic acids, were also rapidly converted to coenzyme A thioesters by purified mitochondria. Acyl-CoA synthetase activities towards stearic, pristanic and phytanic acids using purified mitochondria were, respectively, 87, 65 and 47 nmol/min per mg protein. Purified mitochondrial outer membranes effectively converted fatty acids to coenzyme A thioesters. Mitochondrial outer membrane acyl-CoA synthetase activities towards stearic, pristanic and phytanic acids were, respectively, 164, 172 and 80 nmol/min per mg protein. There was a twofold enrichment of acyl-CoA syn-

Table 1
Marker enzyme activities (nmol/min/mg protein) in liver homogenate, purified mitochondria and mitochondrial outer membranes

Marker enzyme	Liver homogenate	Purified mitochondria	Mitochondrial outer membranes
Monoamine oxidase	4.5	13.6	212.6
α-Ketoglutarate dehydrogenase	3.4	9.0	0.3
Dihydroxyacetone phosphate acyl transferase	2.5	0.7	0.6
Acyl-CoA oxidase	3.8	0.7	0
Uric acid oxidase	4.0	1.7	0

Marker enzyme activities were assayed as described in section 2, and the data in nmol/min/mg protein are presented as an average of two observations. Acyl-CoA oxidase activity was determined using palmitoyl-CoA as a substrate. Dihydroxyacetone phosphate acyltransferase, acyl-CoA oxidase and uric acid oxidase activities were at least fiftyfold higher in purified peroxisomes compared to liver homogenate. The data are representative of three experiments.

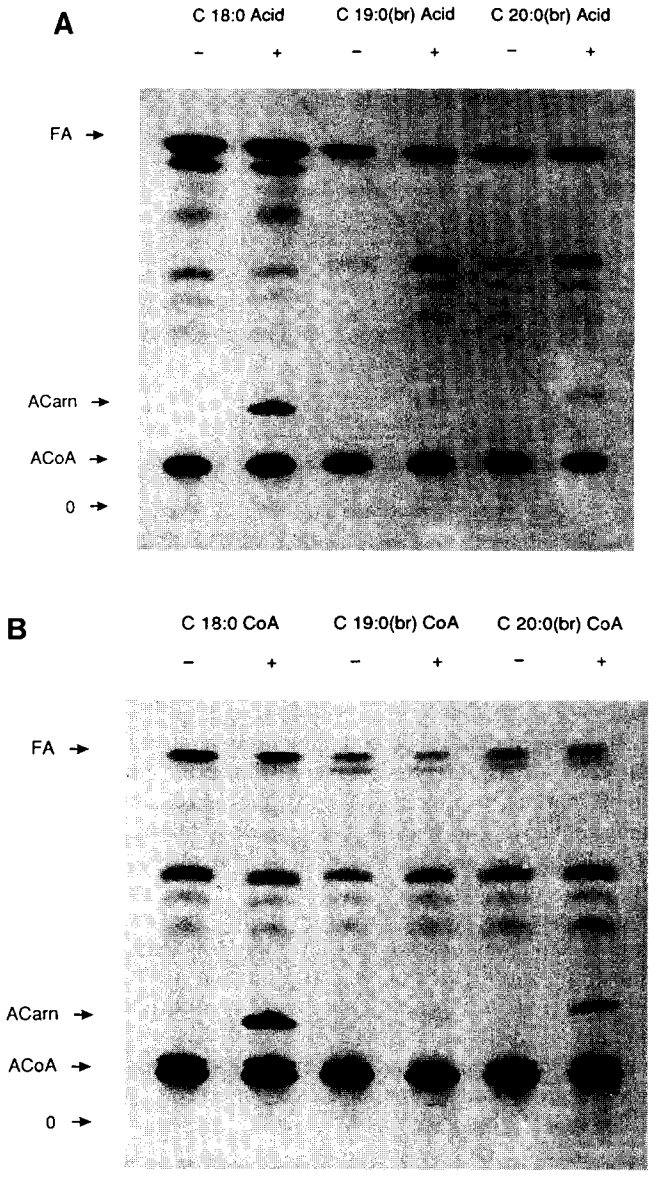

Fig. 1. Autoradiographs of the reaction products formed from free fatty acids and fatty acyl-CoAs by incubations with mitochondrial outer membranes. [1-^{14}C]Fatty acids (A) or [1-^{14}C]fatty acyl-CoAs (B) were incubated (see section 2.4.1) with rat liver mitochondrial outer membranes (4.8 μg protein). The reaction product was chromatographed on TLC silica gel 60 plates and the plates were subjected to autoradiography for 12 days. Fatty acid substrates employed were: C18:0 acid, stearic acid; C19:0 (br) acid, pristanic acid; C20:0 (br) acid, phytanic acid. Lanes with − represent incubations performed without L-carnitine and + represent the incubations in the presence of 2 mM L-carnitine. FA, fatty acid; ACarn, acyl carnitine; ACoA, acyl-CoA; O, origin of applications of reaction products on TLC plates.

thetase activity in mitochondrial outer membranes compared to purified mitochondria, suggesting that the enzyme was partially inactivated during preparation and purification of mitochondrial outer membranes.

Mitochondria contain both CPTI and CPTII, whereas mitochondrial outer membranes contain only CPTI. Therefore, mitochondrial outer membranes were employed for investigations into CPTI activity. Stearic acid, as well as stearoyl-CoA, were

Table 2
Carnitine acyl transferase activity (nmol/min/mg protein) in rat liver mitochondrial outer membranes

Substrate	Acyl carnitine formed from radiolabelled fatty acids or fatty acyl-CoAs		
	Stearate	Pristanate	Phytanate
Free fatty acid	5.3	0	0.2
Fatty acyl-CoA	6.9	0.1	0.2

[1-^{14}C]Fatty acids (5–15 μM) or [1-^{14}C]fatty acyl-CoAs (10–12 μM) were incubated with mitochondrial outer membranes (4.8 μg protein) as described (see section 2.4.1.). Acyl carnitine was separated from acyl-CoA by DEAE-Sephacyl column chromatography [22], and radiolabelled acyl carnitine produced from radiolabelled fatty acid or fatty acyl-CoA was determined. The values, in nmol/min/mg protein, represent an average of two determinations. The experiments were repeated and similar results were obtained.

effectively converted to stearoyl carnitine by mitochondrial outer membranes (Table 2, Fig. 1). Similar results were obtained using palmitic acid and palmitoyl-CoA as substrates (data not given). Pristanic and phytanic acids and their coenzyme A thioesters were poor substrates for the mitochondrial outer membrane enzyme (Table 2, Fig. 1). It is evident that higher enzyme activities were observed with phytanic acid or phytanoyl-CoA as a substrate compared to pristanic acid or pristanoyl-CoA (Table 2, Fig. 1). Quantification of radiolabelled acyl carnitine production from radiolabelled fatty acids or fatty acyl-CoAs was carried out by two different procedures, namely thin layer chromatography (Fig. 1) and DEAE-Sephacyl column chromatography (see section 2, Table 2). The acyl carnitine region from thin layer plates was also scraped and radioactivity was quantified. Quantification of the radioactivity in the acyl carnitine region of the chromatographic plates gave identical results, as described in Table 2 (determined by DEAE-Sephacyl column chromatography).

In order to establish whether phytanic acid can or can not be transported into mitochondria via the carnitine/acyl carnitine transport system, we performed detailed kinetic experiments using unlabelled phytanoyl-CoA and radiolabelled carnitine as substrates, and mitochondrial outer membranes as an enzyme source. For comparison, unlabelled palmitoyl-CoA was employed as a substrate. Palmitoyl carnitine production from palmitoyl-CoA was inhibited by malonyl-CoA (>50% inhibition with 2 μM malonyl-CoA). Significantly lower inhibition by malonyl-CoA was observed when the enzyme assays were performed at 37°C compared to 30°C and at pH 8.0 compared to pH 7.0 (data not given). Significantly lower malonyl-CoA inhibition was observed when fatty acid-free bovine serum albumin was omitted from the incubations. These observations suggest that malonyl-CoA dissociates from CPTI rapidly at higher pH and higher temperature, and the free concentration of palmitoyl-CoA (substrate) in the incubations determine the rate and extent of inhibition by malonyl-CoA.

Under optimal assay conditions and using palmitoyl-CoA as substrate, palmitoyl carnitine production increased with time and the enzyme activity was linear at least up to 10 min. However, under the same conditions with phytanoyl-CoA as a substrate, the enzyme activity was negligible and the activity did not increase with time (Fig. 2A). Palmitoyl carnitine production increased with increasing concentrations of protein, and the reaction was linear, at least up to 7.5 μg protein/assay. Under

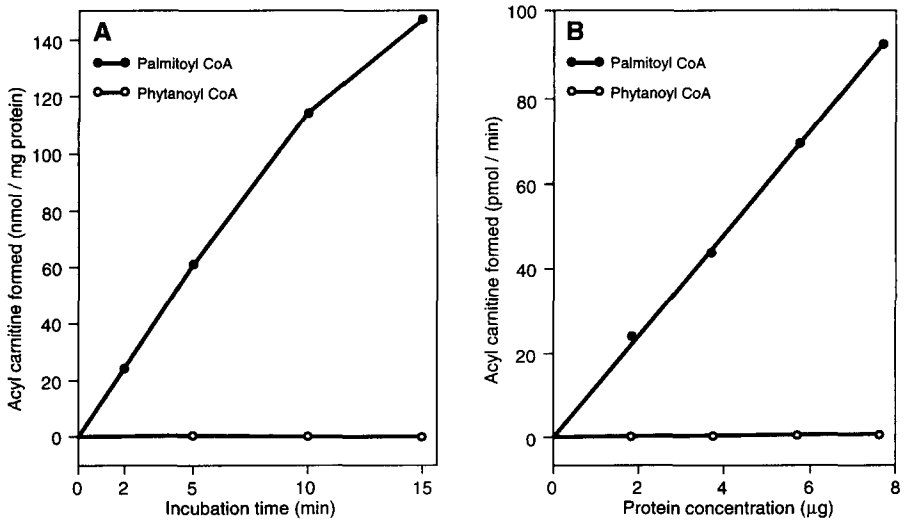

Fig. 2. Time–course and protein curve with palmitoyl-CoA or phytanoyl-CoA as a substrate for carnitine palmitoyltransferase I. Carnitine acyltransferase assays were performed for indicated periods (A) with 5.7 μg of mitochondrial outer membranes or with indicated amounts of mitochondrial outer membranes (B) for 5 min at 30°C (see section 2.4.2). Each point on the graph represents an average of two observations.

the same conditions, phytanoyl carnitine production from phytanoyl-CoA was negligible, and the enzyme reaction did not increase with increased amounts of enzyme protein in the incubations (Fig. 2B).

In order to eliminate the possibility that higher concentrations (100 μM) of phytanoyl-CoA employed might inhibit CPTI, we carried out substrate saturation kinetics with phytanoyl-CoA, as well as palmitoyl-CoA and L-carnitine (Fig. 3A,B). Palmitoyl carnitine production increased with increasing concentrations of L-carnitine or palmitoyl-CoA in the incubations. Typical hyperbolic substrate saturation curves were observed with both L-carnitine and palmitoyl-CoA. However, phytanoyl carnitine production was negligible under these conditions, and the rate of reaction did not change with the increasing concentrations of phytanoyl-CoA in the incubations. Apparent K_m and V_{max} with palmitoyl-CoA and L-carnitine were 55 and 42 μM, and 26 and 21 nmol/min per mg protein, respectively.

It is known that bovine serum albumin binds fatty acids and fatty acyl-CoAs. It is possible that bovine serum albumin added to incubations may tightly bind phytanoyl-CoA, and that albumin-bound phytanoyl-CoA may not be available to the enzyme. To address this question, we excluded bovine serum albumin from the incubations and repeated the substrate saturation kinetic experiments. Again, a hyperbolic palmitoyl-CoA saturation curve was observed at the lower concentrations of the substrate, and higher concentrations of the substrate were inhibitory (Fig. 3C), possibly due to detergent action. Apparent K_m and V_{max} with palmitoyl-CoA were 18 μM and 77 nmol/min/mg protein, respectively. CPTI activity with phytanoyl-CoA was negligible at all the substrate concentrations examined (Fig. 3C).

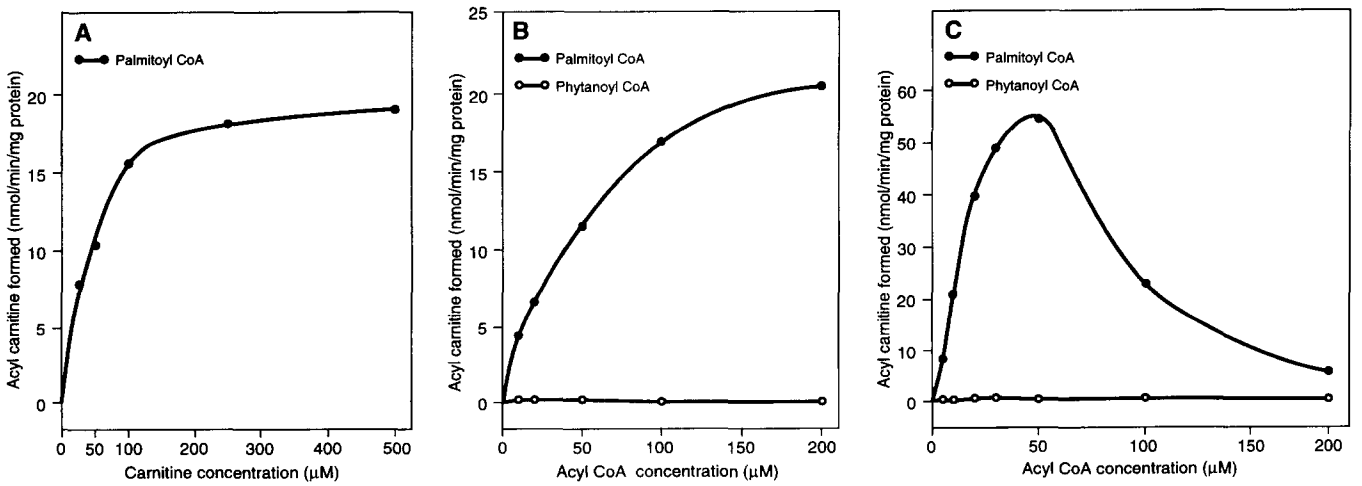

Fig. 3. Substrate saturation kinetic studies with carnitine palmitoyl transferase I. Mitochondrial outer membranes (7.6 μg protein) were incubated at 30°C for 5 min. with 100 μM palmitoyl-CoA and indicated concentrations of L-carnitine (A), 250 μM L-carnitine and indicated concentrations of palmitoyl-CoA or phytanoyl-CoA (B,C). Equimolar concentrations of acyl-CoA and fatty acid free bovine serum albumin were maintained in the incubations (A,B) or fatty acid free bovine serum albumin was omitted from the incubations (C) and acyl carnitine production was measured (see Method II of carnitine acyltransferase assay). Each point on the graph represents an average of two observations.

4. Discussion

The data presented clearly indicate that both the unbranched and branched long chain fatty acids are activated to acyl-CoAs by the mitochondrial outer membrane acyl-CoA synthetase. It is not clear at present whether the branched chain fatty acids are activated by a distinct branched chain acyl-CoA synthetase or by a non-specific long chain acyl-CoA synthetase located in the mitochondrial outer membranes. Pristanic acid with an α-methyl group can be degraded by β-oxidation, whereas phytanic acid with a β-methyl group can only be degraded by α-oxidation. The naturally occurring branched chain fatty acids containing either an α-methyl or a β-methyl group can be effectively activated to acyl-CoA by mitochondrial outer membrane acyl-CoA synthetase. However, both these fatty acids are poor substrates for CPTI (Table 2, Figs. 1–3). It is well accepted that long chain fatty acids are transported across the mitochondrial membranes via a carnitine/acyl carnitine transport system (see section 1). Our data clearly demonstrate that CPTI is highly specific for unbranched chain fatty acids. The fact that both the branched chain fatty acids, namely pristanic and phytanic acids, can not be converted to acyl carnitine by CPTI clearly indicates that pristanic and phytanic acids cannot be transported into mitochondria via the carnitine/acyl carnitine transport system. Thus it is unlikely that pristanic and phytanic acids, the two branched chain fatty acids, are oxidized in rat liver mitochondria. Recent studies from our laboratory [17,26] provide evidence that in human skin fibroblasts, pristanic acid oxidation occurs mainly in peroxisomes.

In contrast to β-oxidation, the exact intracellular localisation of α-oxidation is poorly understood. Recent studies in human liver and skin fibroblasts [27–29] suggest that α-oxidation of phytanic acid is peroxisomal, whereas in rat liver the oxidation is mitochondrial [28]. However, previous studies in rat, human and monkey liver suggested that α-oxidation occurs in mitochondria [30–33]. Also, the report that phytanic acid oxidation is defective in patients with cytochrome *c* oxidase deficiency [34] indicates that mitochondria are involved in the production of radiolabelled CO_2 from [1-^{14}C]phytanic acid. A report that α-oxidation of phytanic acid is mainly microsomal in rat liver [35] further adds to the confusion. In all of these studies, radiolabelled CO_2 production from [1-^{14}C]phytanic acid was taken as evidence of α-oxidation. Furthermore, α-oxidation activities measured were very low [27–35]. Recent studies from our laboratory [36] provide evidence that formic acid is the major α-oxidation product in human skin fibroblasts. Formic acid production is deficient in Zellweger syndrome fibroblasts, indicating that formic acid production from phytanic acid is peroxisomal, although the exact mechanism of α-oxidation of phytanic acid is poorly understood at present. The evidence presented herein clearly indicate that phytanic acid α-oxidation in rat liver is unlikely to be mitochondrial. Further studies are required to understand the exact mechanism(s) of phytanic acid oxidation and to clarify the intracellular site of α-oxidation.

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