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CDP-diacylglycerol synthesis in rat liver mitochondria

Amy Y.P. Mok, Gordon E. McDougall and William C. McMurray

University of Western Ontario, Department of Biochemistry, London, Ont., Canada

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CDP-diacylglycerol for polyglycerophosphatide biogenesis can be synthesized within rat liver mitochondria. This membrane-associated enzyme was predominantly located in the inner mitochondrial membrane. GTP had a significant effect in activating the microsomal CDP-diacylglycerol synthase, especially if the microsomes were preincubated with GTP in the presence of phosphatidic acid. This stimulatory effect of GTP on the microsomal enzyme was not detected in the mitochondrial fractions. The enzymes could be solubilized from the membrane fractions using CHAPS, and the detergent-soluble activity partially restored by addition of phospholipids. Mitochondrial and microsomal CDP-diacylglycerol synthase activity could be completely separated by anion-exchange column chromatography. The mitochondrial and microsomal CDP-diacylglycerol synthases appear to be two distinct enzymes with different localization and regulatory characteristics.

CDP-diacylglycerol; Cytidylyltransferase; Phosphatidic acid; Mitochondria; Solubilization

1. INTRODUCTION

The liponucleotide, CDP-diacylglycerol, is synthesized from CTP and phosphatidic acid catalyzed by the enzyme CDP-diacylglycerol synthase (CTP: phosphatidate cytidylyltransferase EC 2.7.7.41) [1]. This enzyme activity has been found in both bacteria and eukaryotes [2–4]. CDP-diacylglycerol is an obligatory intermediate compound in the biosynthesis of the anionic phospholipids such as cardiolipin, phosphatidylglycerol and phosphatidylinositol [5]. The endoplasmic reticulum is the major site for the biosynthesis of phosphatidylinositol in mammals, and other polyglycerophosphatides and cardiolipin are nearly exclusively synthesized in mitochondria [6,7].

Biosynthesis of CDP-diacylglycerol occurs in both the mitochondrial and microsomal fractions in rat liver [3,8], with the majority of activity associated with the microsomes [9]. However, there are conflicting reports in the literature concerning the actual distribution of the synthase activity in rat hepatocytes. Hostetler and Van den Bosch [8] and Jelsema and Morrè [3] based on marker enzyme studies concluded that rat liver mitochondria contain intrinsic CDP-diacylglycerol synthase. Davidson and Stanacev [10] discounted this activity in mitochondria as due to the microsomal contamination.

Correspondence address: A.Y.P. Mok, University of Western Ontario, Department of Biochemistry, London, Ont., Canada.

Abbreviations: CDP-diacylglycerol, cytidine diphosphate diacylglycerol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanosulfonate; PA, phosphatidic acid; DTT, DL-dithiothreitol; BSA, bovine serum albumin; βME, β-mercaptoethanol.

This report describes comparative studies of CDP-diacylglycerol synthase activities in isolated mitochondria and microsomes from rat liver. It was found that both mitochondria and microsomes were able to synthesize CDP-diacylglycerol from CTP and phosphatidic acid. However, there are significantly different properties in the enzyme activities found in mitochondria and microsomes. A clear separation of the two forms of the enzyme from the mitochondria and microsomes will also be documented.

2. MATERIALS AND METHODS

2.1. *Materials*

Male Wistar rats (200–250 g) were used for all experiments. The 5-[^{3}H]cytidine-5'-triphosphate was purchased from NEN-Dupont with a specific activity of 27 Ci/mmol. Tricine, BSA, CHAPS, and CTP (type I from muscle) were obtained from Sigma Chemical Company (St. Louis, MO). Most of the phospholipids were purchased from Serdary Research Laboratory (London, Ont.). Atlas G-3634A was obtained from Atkemix Inc. (Brantford, Ont.). All other materials were purchased from Canlab and were of reagent grade or better.

2.2. *Preparation of enzymes*

Mitochondrial and microsomal fractions were prepared from rat liver as described by McMurray and Dawson [11]. The mitochondrial membranes were prepared by sequential hypotonic and hypertonic buffer salt extraction as previousiy described [12,13]. The submitochondrial fractions were obtained by sonication of whole mitochondria as described by Sottocasa et al. [14]. The CDP-diacylglycerol synthases were extracted from both subcellular fractions by CHAPS based on the procedure described by Sparrow and Raetz [2]. CHAPS at 0.5% was normally used in the extraction procedure. The determination of the relative specific activity for CDP-diacylglycerol synthase in the non-solubilized membrane fragments revealed that this enzyme was not completely extracted. Generally, the recovery of CDP-diacylglycerol synthase activity in the detergent-solubilized fractions was greater than 50%.

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2.3. *Assay of CDP-diacylglycerol synthase*

The activity of CDP-diacylglycerol synthase was measured by determining the rate of [^{3}H]CTP incorporated into the lipid fraction. The assay condition was a modification of Bishop and Strickland's method [4]. The reaction mixture in a final volume of 0.3 ml contained: 0.23 mM phosphatidic acid (prepared according to Bishop and Strickland [4]), 50 mM maleate buffer pH 7.0, 30 mM $MgCl_2$, 16 μg Atlas G-3634A, 0.5 mM DTT, 2.5 mM 5-[^{3}H]CTP (2 μCi) and 0.4-0.6 mg protein.

The assay mixture was preincubated for 3 min at 37°C then [^{3}H]CTP was added to initiate the reaction. The reaction time ranged from 5 to 10 min at 37°C. For routine assays, at the end of 5 min incubation the reaction was terminated by the addition of 4 ml $CHCl_3$/CH_3OH (1:1, v/v). A two phase system was attained by adding 1.8 ml of 0.1 N HCl containing 1 M NaCl. The upper phase which contained the unreacted CTP was discarded. The lower phase was washed three times with 1.8 ml 0.1 N HCl containing 1 M NaCl and then 1 ml of the washed lower phase was transferred into a counting vial and air-dried completely for radioactivity determination.

2.4. *Preparation of GTP-activated CDP-diacylglycerol synthase*

The activation of CDP-diacylglycerol synthase by GTP was conducted as described by Liteplo and Sribney [15]. A 0.5 ml portion of mitochondria or microsomes (about 15 mg protein) was incubated in 100 mM Tris-HCl buffer (pH 7.2), 4 mM DTT, 2 mM GTP, 20 mM $MgCl_2$ and 1 mM phosphatidic acid from egg lecithin (emulsified in 0.5 mg Atlas/ml) in a final volume of 1 ml. The control was incubated with the same buffer but in the absence of 2 mM GTP. After 20 min incubation at 37°C, the membrane fractions were isolated by centrifugation at 48,000 rpm (165,000 × g) for 45 min at 4°C. The pellets were resuspended in 0.5 ml of 25 mM Tris-HCl buffer (pH 7.4). The suspensions were used for determination of CDP-diacylglycerol synthase activity as described above, and the protein concentration was determined by the Lowry method [16].

2.5. *Anion-exchange chromatography*

The solubilized fractions from mitochondria and microsomes were partially purified by ion-exchange chromatography as described by Sparrow and Raetz [2]. The solubilized fraction was applied to a DEAE Bio-Gel A (purchased from Bio-Rad) 1.5 × 18 cm column which had been equilibrated with the buffer containing: 10 mM Tris (pH 7.0), 10% glycerol, 0.3% CHAPS and 1 mM DTT or 10 mM βME. A peristaltic pump was used to facilitate a steady column flow rate of 14 ml/h. Approximately 50 ml of the same buffer was used to wash the column (or until no more protein was detected in the eluate) then a linear sodium chloride gradient from 0–0.8 M in a total volume of 80 ml was applied to elute the adsorbed protein from the column. The eluate was collected in 2.0 ml fractions and the protein profile was measured by the Bradford method [17] (using the assay kit provided by Bio-Rad Laboratories). The recovery of the CDP-diacylglycerol synthase activity was low (less than 5%) due to the instability of the enzyme.

2.6. *Other methods*

The cross contamination of mitochondrial and microsomal fractions was determined by measuring the specific activity of the marker enzymes: succinate cytochrome c reductase for mitochondria [11,18] and NADPH cytochrome c reductase for microsomes [14]. Monoamine oxidase, a marker enzyme for outer mitochondrial membranes, was assayed by the method of Wurtman and Axelrod [19].

3. RESULTS

3.1. *Subcellular distribution of the enzyme*

In order to determine if rat liver mitochondria contained intrinsic CDP-diacylglycerol synthase activity, the enzyme activities from both subcellular fractions were measured under optimal conditions and compared to the mitochondrial and microsomal marker enzyme activities (succinate cytochrome c reductase and NADPH cytochrome c reductase respectively) from the same sample. The mitochondrial fraction contained less than 5% of the marker enzyme activity present in the microsomal fraction. The mitochondrial contamination in the microsomal fraction was found to be negligible (data not shown).

Approximately 83% of the enzyme activity in the mitochondrial fractions was recovered. The majority of the enzyme activity copurified with the inner mitochondrial membrane-enriched fraction with a relative specific activity twice that in whole mitochondrial fractions (Table I). The enriched outer membrane fraction contained only 1% of the total recovered activity and there was no activity of the enzyme in the soluble protein fraction. CDP-diacylglycerol synthase activity in mitochondria did not copurify with the microsomal marker

Table I

Subfractions of mitochondrial membranes

Enzymes	Whole mitochondria	Inner membranes	Outer membranes	Soluble
Succinate-cyt c reductase (IM)	70.5 ± 1.4 (1.0)	95.1 ± 2.3 (1.349)	0 (0)	0 (0)
NADPH-cyt c reductase (MS)	3.0 ± 0.4 (1.0)	1.10 ± 0.02 (0.367)	10.5 ± 0.13 (3.5)	5.5 ± 0.09 (1.83)
Monoamine oxidase (OM)	1267.9 ± 86.1 (1.0)	867.2 ± 141.8 (0.684)	1771.2 ± 28.4 (1.397)	194.8 ± 35.7 (0.153)
CDP-DG synthase	170.5 ± 12.2 (1.0)	340.2 ± 16.3 (1.995)	40.1 ± 3.4 (0.2352)	0 (0)

Submitochondrial distribution of CDP-diacylglycerol synthase. Specific activities for succinate- and NADPH-cytochrome c reductase are μmol/min/mg; for monoamine oxidase, nmol/min/mg; and for CDP-diacylglycerol synthase, pmol/min/mg. Numbers in parentheses represent the relative specific activities with respect to whole mitochondria. Specific activities are the mean of 3 observations ± S.D. (IM, inner mitochondrial membrane; MS, microsomes; OM, outer mitochondrial membrane).

enzyme, supporting the finding that the incorporation of CTP into CDP-diacylglycerol in the mitochondrial inner membrane fraction could not be due to the microsomal contamination.

3.2. *Comparative studies of mitochondrial and microsomal CDP-diacylglycerol synthase*

Intact, freshly prepared mitochondria produced a sigmoidal response in the CTP substrate curve (data not shown). Since this sigmoidal response was abolished in mitochondrial fractions which had been subjected to hypotonic and hypertonic treatment or to freezing and thawing, it might be due to the requirement for transport of the exterior CTP through the mitochondrial membrane barrier to the enzymatic site. Microsomal fractions never showed any sign of a sigmoidal response.

Mitochondrial CDP-diacylglycerol synthase attained maximal activity at a relatively low phosphatidic acid concentration (about 0.1 mM). Unlike mitochondria, microsomes showed a linearity even up to 0.4 mM phosphatidic acid (data not shown). Apparently, the availability of phosphatidic acid may be a limiting factor for CDP-diacylglycerol synthesis in microsomal membranes. The addition of GTP in the assay mixture specifically and significantly stimulated CDP-diacylglycerol synthesis in the microsomal fraction but this stimulation was not observed in the mitochondrial fraction. This stimulatory effect of GTP on CDP-diacylglycerol synthase in the microsomal fraction was more pronounced if the microsomes were pretreated with GTP as described in section 2. The treatment of the microsomal fraction with GTP stimulated the rate of production of CDP-diacylglycerol at least two-fold when compared with the untreated microsomes (Table II). This stimulatory effect was consistently observed in the GTP-treated microsomes either in the presence or absence of GTP in the assay mixture. Unlike microsomes, the treatment with GTP seemed to inhibit the CDP-diacylglycerol synthase activity in the mitochondrial fraction. The treatment with GTP lowered the K_m value for phosphatidic acid in the microsomal fraction (data not shown). These observations suggested that treatment with GTP altered the apparent affinity for phosphatidic acid of mitochondrial and microsomal CDP-diacylglycerol synthases in different ways.

Table II

Effect of GTP pretreated mitochondria and microsomes in CDP-diacylglycerol synthesis

		CDP-diacylglycerol synthase	
		Assay conditions	
Cell fraction	GTP-pretreatment	No GTP	1 mM GTP added
Mitochondria	−2 mM GTP	109.5 ± 1.1	86.4 ± 4.9
	+2 mM GTP	45.5 ± 0.2	44.7 ± 0.4
Microsomes	−2 mM GTP	130.5 ± 1.7	230.1 ± 1.1
	+2 mM GTP	372.3 ± 0.6	317.0 ± 0.2

Comparison of the CDP-diacylglycerol synthesis on GTP pretreated mitochondria and microsomes as described in the Methods section. The assay condition was described in the method either in the presence of 1 mM GTP or in the absence of GTP.

3.3. *Anion-exchange chromatography*

As the enzyme activity was inhibited by most detergents they were unsuitable for extracting CDP-diacylglycerol synthase from the mitochondrial or microsomal membranes. CHAPS was found to be the only useful detergent for solubilizing the enzyme, although a significant loss of enzyme activity occurred in the solubilized fractions, and once the enzyme was extracted by CHAPS it appeared to be relatively unstable. The instability of the solubilized CDP-diacylglycerol synthase made it very difficult to purify. Mitochondrial and microsomal CDP-diacylglycerol synthases behaved very differently on anion-exchange chromatography (DEAE Bio-gel A column). The activity of most of the mitochondrial CDP-diacylglycerol synthase did not adsorb to the column and came out in the wash fraction. Unlike the mitochondrial enzyme, microsomal CDP-diacylglycerol synthase was completely adsorbed onto the DEAE Bio-gel A column and required 0.3 M sodium chloride to elute it from the column (Fig. 1A,B). When a mixture of CHAPS extracts from mitochondria and microsomes was applied onto the same column, two distinct activity peaks were detected, one in the wash volume and the other in the 0.3 M sodium chloride eluate. Thus, mitochondrial and microsomal CDP-diacylglycerol synthases could be cleanly separated by an anion-exchange column (Fig. 1C).

4. DISCUSSION

The CDP-diacylglycerol synthase of mammalian cells and prokaryotes has been extensively studied; CDP-diacylglycerol synthase has been detected in different animal tissues [4], and it was shown to be a key enzyme for the synthesis of membrane phospholipids in *Escherichia coli* [20]. Microsomes are definitely the major site for the synthesis of CDP-diacylglycerol in eukaryotes where the availability of CDP-diacylglycerol may control the biosynthesis of phosphatidylinositol. The synthesis of phosphatidylinositol is exclusively in microsomes, and apparently the phosphatidylinositol in mitochondria is renewed by an exchange mechanism between mitochondria and microsomes [11].

CDP-diacylglycerol is also an important intermediate for the synthesis of phosphatidylglycerol and cardiolipin (diphosphatidylglycerol) in mitochondria [8,10]. Not much research has been done on CDP-diacylglycerol synthase in animal mitochondria, although the en-

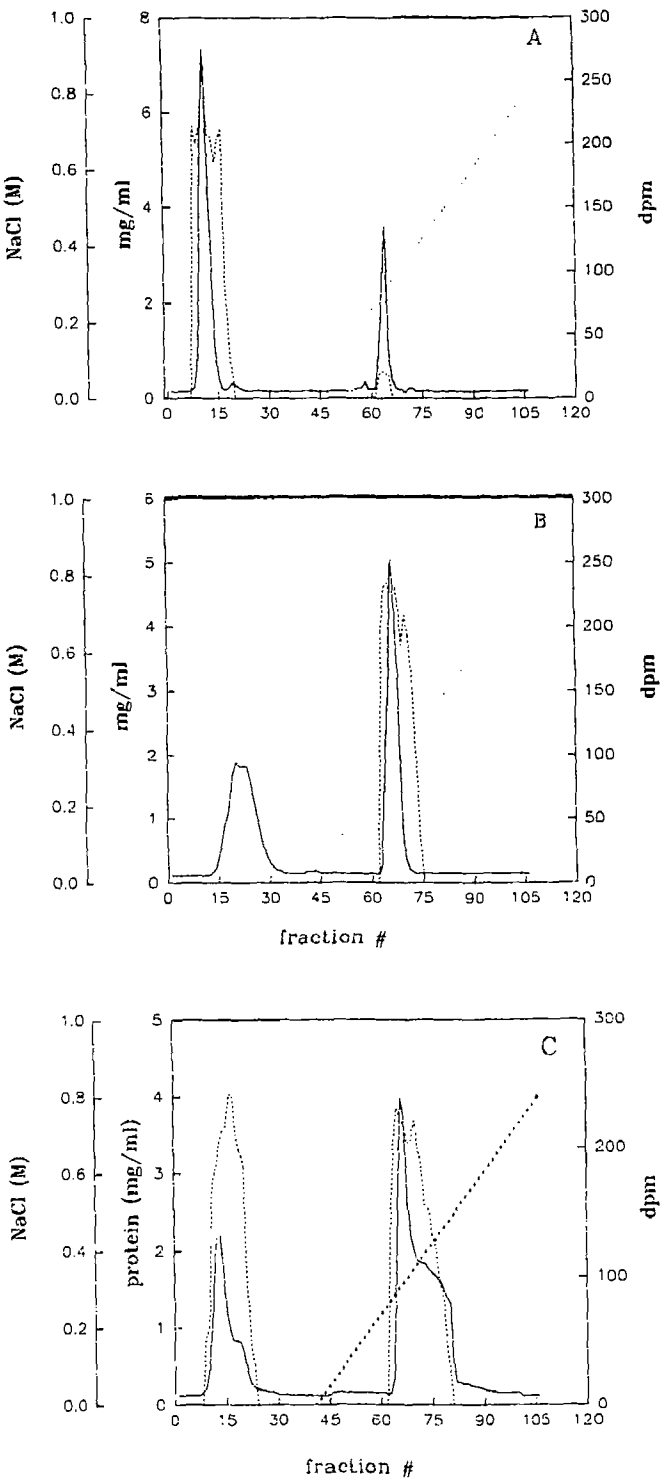

Fig. 1. Partial purification of CDP-diacylglycerol synthase by DEAE Bio-gel A column chromatography. (A) Solubilized mitochondrial fraction, (B) solubilized microsomal fraction, and (C) mixture of solubilized mitochondrial and microsomal fractions were applied to the DEAE Bio-gel A column and eluted with a sodium chloride gradient. Fractions were collected and analyzed for protein (———) and CDP-diacylglycerol synthase activity (----) as described in the methods section. The sodium chloride gradient is shown by the dotted line (fractions 40–110).

zyme has been purified from yeast mitochondria [21]. A small but significant activity of CDP-diacylglycerol synthase is found in mammalian mitochondria [3,8]. However, Davidson and Stanacev [10] showed that the distribution of CDP-diacylglycerol synthase activity paralleled the distribution of microsomal marker enzyme activity in rat liver. Van Golde et al. [22] and Rüstow et al. [23] found some minor activity of synthase in rat liver mitochondrial fractions, but the existence of a true mitochondrial CDP-diacylglycerol synthase free of microsomal contaminants was not clear. This report studied some properties of the mitochondrial CDP-diacylglycerol synthase and compared them with those of the microsomal enzyme.

Intrinsic mitochondrial synthase activity is evidenced by the results with marker enzymes. Our results agree with the finding by Bishop and Strickland for beef brain [4], although the distribution of CDP-diacylglycerol synthase in mitochondria has varied in other studies. It was not clear whether CDP-diacylglycerol synthase is one enzyme protein having a bilocal distribution or whether two isoenzymes exist, one for each membrane. Jelsema and Morrè [3] detected CDP-diacylglycerol synthase activity in both the outer and inner mitochondrial membranes of rat liver. A contradiction with this result by Hostetler and Van den Bosch [8] showed that CDP-diacylglycerol synthase activity was predominantly in the inner mitochondrial membrane, and the activity of the enzyme detected in the outer membrane was due to the microsomal contamination. Our results are consistent with the latter findings. With the optimal assay system we used, mitochondrial CDP-diacylglycerol synthase copurified with the inner membrane enriched fraction. The presence of the enzyme in the inner mitochondrial membrane could also explain the observation of sigmoidal responses of whole mitochondria to increasing CTP concentrations. Treatment by sequential hypotonic–hypertonic washing or sonication, which would disrupt the integrity of the mitochondrial membrane permeability barrier abolished the sigmoidal responses. These results suggest that CDP-diacylglycerol synthase may be accessible only to intramitochondrial CTP in intact mitochondria.

Guanine nucleotides play a key role in a number of metabolic processes including protein biosynthesis [24], tubulin polymerization [25] and the regulation of adenylate cyclase activity [26]. GTP has been found to enhance the activity of microsomal CDP-diacylglycerol synthase [15,27,28]. Liteplo and Sribney [15,27] showed that the stimulatory effect of GTP was observed only in the presence of phosphatidic acid, and the stimulation of the enzyme was specific to GTP. They also showed that the magnitude of the GTP-stimulatory effect on CDP-diacylglycerol synthase was influenced by the fatty acid composition of phosphatidic acid [29]. They suggested that the binding of phosphatidic acid to the enzyme probably induced a conformational change,

which allowed the stimulatory action of GTP to be observed. GTP decreased the K_m value for phosphatidic acid with the rat liver microsomal synthase. In our studies mitochondrial synthase responded differently from microsomal enzyme to extraneous GTP. The treatment of the mitochondrial fraction under the same conditions which activated the microsomal synthase by GTP apparently decreased the synthesis of CDP-diacylglycerol. These results showed that GTP may play opposite roles in the regulation of anionic phospholipid synthesis in mitochondria and microsomes.

Phosphatidylglycerol and cardiolipin are exclusively synthesized in mitochondria [6,7] and the low level of CDP-diacylglycerol found in mammalian tissues suggests that the synthesis of CDP-diacylglycerol could be rate limiting in the biosynthesis of these polyglycerolipids [30]. It is now clear that CDP-diacylglycerol definitely can be synthesized in mitochondria. Even though this inner membrane enzyme activity is low in comparison to the microsomal activity, it may be sufficient for in situ conversion to mitochondrial phosphatidylglycerol and cardiolipin [31,32]. It is known that the formation of phosphatidic acid may occur in mitochondria and microsomes by the acylation of glycerophosphate. This reaction in mitochondria is distinct from that in microsomes [13,33]. This suggests that under conditions in vivo, mitochondrial and microsomal CDP-diacylglycerol synthases may be accessible to different pools of phosphatidic acid, CTP and magnesium ions. All evidence provided in this report suggests that mitochondrial and microsomal CDP-diacylglycerol synthases appear to be distinct isoforms, differently regulated and located in different compartments of the cell, with the majority of enzyme activity occurring in the microsomal fraction.

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