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Extraction and Stabilization of Mammalian CDP-Diacylglycerol Synthase Activity

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CDP-diacylglycerol synthase, also known as CTP: phosphatidic acid cytidylyltransferase (EC 2.7.7.41), is thought to be the rate-limiting enzyme in the synthesis of the inositol phospholipids, phosphatidylglycerol and cardiolipin. Its role in inositol phospholipid synthesis suggests its potential as a regulator of signal transduction as well. Although the mammalian cDNA for the synthase has recently been cloned, attempts to purify this enzyme from a mammalian source have been unsuccessful due to its lability in detergents. We report here the extraction and stabilization of CDP-diacylglycerol synthase from rat liver. Using a buffer containing 2M KCl, we were able to extract virtually all of the activity from microsomal membranes. This extract was stable indefinitely at −72°C and for at least 24 hrs at 4°C. Incubation at room temperature for 24 hours resulted in the loss of more than half the activity. All detergents tested destroyed the activity. The activity was dependent on both substrates (phosphatidic acid and CTP) as well as on $MgCl_2$, and inhibited by the product, CDP-diacylglycerol. Addition of GTP enhanced the activity approximately 2 fold, and bovine serum albumin increased activity by 6 fold.

The phospholipid synthetic enzyme, CDP-diacylglycerol synthase (CDS), transfers a CDP moiety from CTP to phosphatidic acid (PtdOH) to form CDP-diacylglycerol (CDP-DG). This CDP-DG can then be converted to phosphatidylinositol (PtdIns), phosphatidylglycerol (PtdG) or cardiolipin (CL). In most tissues, PtdIns accounts for most of the product (1). The activity is localized primarily in the endoplasmic reticulum, and, to a lesser extent, in the mitochondria (2). The realization that PtdIns plays an important regulatory function in its role as a precursor of the signal-transducing compounds, inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DG), has increased interest in CDS. Although the mammalian form of this enzyme has been a target of investigation for over 30 years (2-8), studies have been hampered by an inability to successfully extract the activity from its native membrane environment in a stably active form.

Recently, the cDNA for both the human and rat enzyme has been cloned, and the amino acid sequence deduced (9-11). However, attempts to purify the human enzyme from cells overexpressing the activity were unsuccessful, again due to its lability (9). We report here the successful extraction and stabilization of CDS from rat liver.

MATERIALS AND METHODS

Rat livers were obtained at the time of euthanasia without regard to sex or age. The livers were frozen at −80°C until further use. The detergent, Hecameg (6-0-(N-heptylcarbamoyl)-methyl-D-glucopyranoside), was purchased from Vegatec (Villejuif, France). Phosphatidic acid (dioleoyl) and CDP-diacylglycerol (didecanoyl) were purchased from Serdary Labs. Tritiated CTP (19.4 Ci/mmole) was obtained from American Radiolabeled Chemicals. Bovine serum albumin (BSA), fatty acid free, was from Sigma, Aquasol 2 was purchased from New England Nuclear. The protein assay kit was purchased from BioRad.

Tissue homogenization. Rat livers were minced and homogenized in 4-5 volumes of 0.25M sucrose with a Polytron homogenizer. All procedures were carried out at 4°C unless stated otherwise. The homogenate was centrifuged at 10,000 × g for 20 minutes to remove nuclei, mitochondria and cell debris. The resulting supernatant (10KS) was centrifuged at 100,000 × g for 60 minutes, and the pellet from this step was resuspended in either Buffer A (50mM glycylglycine, 20% glycerol, pH 7.2) or Buffer E (50mM glycylglycine, 20% glycerol, 2MKCl, pH 7.2). The material resuspended in Buffer A is referred to as the microsomal pellet (MP). The material resuspended in Buffer B (referred to as 100KP) was stored at −75°C overnight and subsequently centrifuged at 100,000×g for 60 minutes. The supernatant resulting from this centrifugation is referred to as the salt extract (SEX). All fractions were stored at −75°C. Protein concentrations were determined using the method of Bradford (12).

Enzyme assay. CDS activity was assayed by monitoring the incorporation of radioactive CTP into CDP-DG in the presence of PtdOH.

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TABLE I

Extraction of CDS Activity from Rat Liver Microsomes

Fraction	[Protein] (mg/ml)	Volume (ml)	Specific activity (pmol/min/mg)	Total activity
		Experiment 1		
10KS	38	25	30	28,500
MP	7.5	15	334	37,575
SEX	6.7	15	420	42,172
		Experiment 2		
10KS	38	25	26	24,700
MP	5.6	15	392	33,053
SEX	4.3	15	490	31,408

Rat livers subcellular fractions were prepared as described in the text. Abbreviations are as follows: 10KS = postmitochondrial supernatant; MP = microsomal pellet; SEX = salt extract of the microsomal pellet.

The assay mixture contained the following: 50mM glycylglycine buffer, pH.7.2, 0.7 mM PtdOH, 1mM CTP, 2μCi [^{3}H]CTP, 50μM $MgCl_2$, 1mM GTP, 0.2MKCl, 6mg/ml BSA and enzyme in a final volume of 100μl. The ingredients (minus $MgCl_2$) were preincubated at 37°C for 10 minutes. $MgCl_2$ was added and the incubation continued for 30 additional minutes. The reaction was terminated by the addition of 0.5ml acidic methanol. Water (O.3ml) and chloroform (1.0ml) were added to each tube, followed by vortexing and centrifugation in a Serofuge (Clay-Adams) to separate the layers. An aliquot of the lower, chloroform layer was transferred to a scintillation vial, dried down, and the radioactivity quantitated after the addition of 8 ml of Aquasol 2. The assay was determined to be linear with time (up to 60 minutes) and protein concentration (20 to 200 μg of SEX per tube). All assays were done in duplicate, with variations averaging 8%.

RESULTS

Extraction and stabilization of CDS. Initial attempts to extract CDS with detergents were unsuccessful. Detergents tested included Hecameg, Zwittergent 3-14, CHAPS, deoxycholate and Triton X-100. All detergents inhibited CDS activity. For example, at very low concentrations (0.01%) of Zwittergent, the activity was inhibited by 22% and at 0.1%, only 5% of the activity remained. Normally, 0.3 to 0.5% Zwittergent is used for solubilization. Attempts to extract the enzyme with high salt concentrations were successful. Table 1 illustrates the results from two separate experiments. Treatment of the 100KP fraction with 2M KCl resulted in extraction of an average of 83% of the microsomal protein and virtually all of the enzyme activity. Similar results were seen in several additional experiments. Figure 1 demonstrates the stability of the KCl extract (SEX). Samples were incubated overnight at 4°C and 22°C. Activity in the salt extract was then compared with that in the unextracted pellet. The extracted activity was at least as stable as the microsomal activity. Both retained full activity at 4°C for 24 hours; however, at room temperature (22°C), both fractions lost activity.

Characterization of salt-extracted CDS. The CDS activity present in SEX is dependent upon addition of the substrates, PtdOH and CTP (figures 2A and 2B). The Km for CTP is 1.2M, while that calculated for PtdOH is 7.4mM. Virtually no activity is observed in the absence of $MgCl_2$ (figure 2C). Addition of 6mg/ml of bovine serum albumin (fatty acid free) to the assay mix increased the activity by approximately 6 fold (figure 2D). Addition of the product, CDP-DG, significantly inhibited the activity (figure 2E); however, neither inositol 1,4,5-trisphosphate (12mM) nor phosphatidylinositol 4,5-bisphosphate (26-74 mol%) inhibited CDS activity (data not shown).

Effect of GTP on CDS activity. It has been previously reported that rat liver microsomal CDS activity is stimulated by GTP (6). Figure 3 illustrates that the KCl extract retains this GTP sensitivity; however, the stimulation by GTP is greater in the microsomal preparation (195% of control) than in the salt extract (151% of control). Several additional experiments yielded similar results.

DISCUSSION

Evidence suggests that CDS activity is pivotal in the functioning of the cell. Since the concentration of its product, CDP-DG, is much lower than that of its substrate PtdOH (13), it has been suggested that the CDS reaction is rate-limiting in the synthesis of PtdIns, PtdG and CL. The central role of CDS in the synthesis of PtdIns further suggests that the enzyme activity is crucial in the generation of a variety of lipid-derived second messengers, and there are experimental data to support the participation of CDS in the resynthetic, or compensatory, phase of the

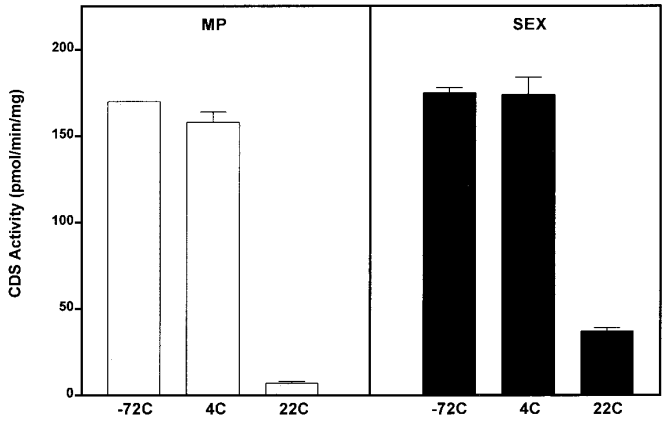

FIG. 1. Stability of CDS Activity. The microsomal pellet (MP) and salt extract (SEX) were prepared as described in text. An aliquot of each was stored at the temperature indicated for 24 hrs. CDS activity was then detrmined as described in the text. Values represent the means ± 1SD of duplicate samples.

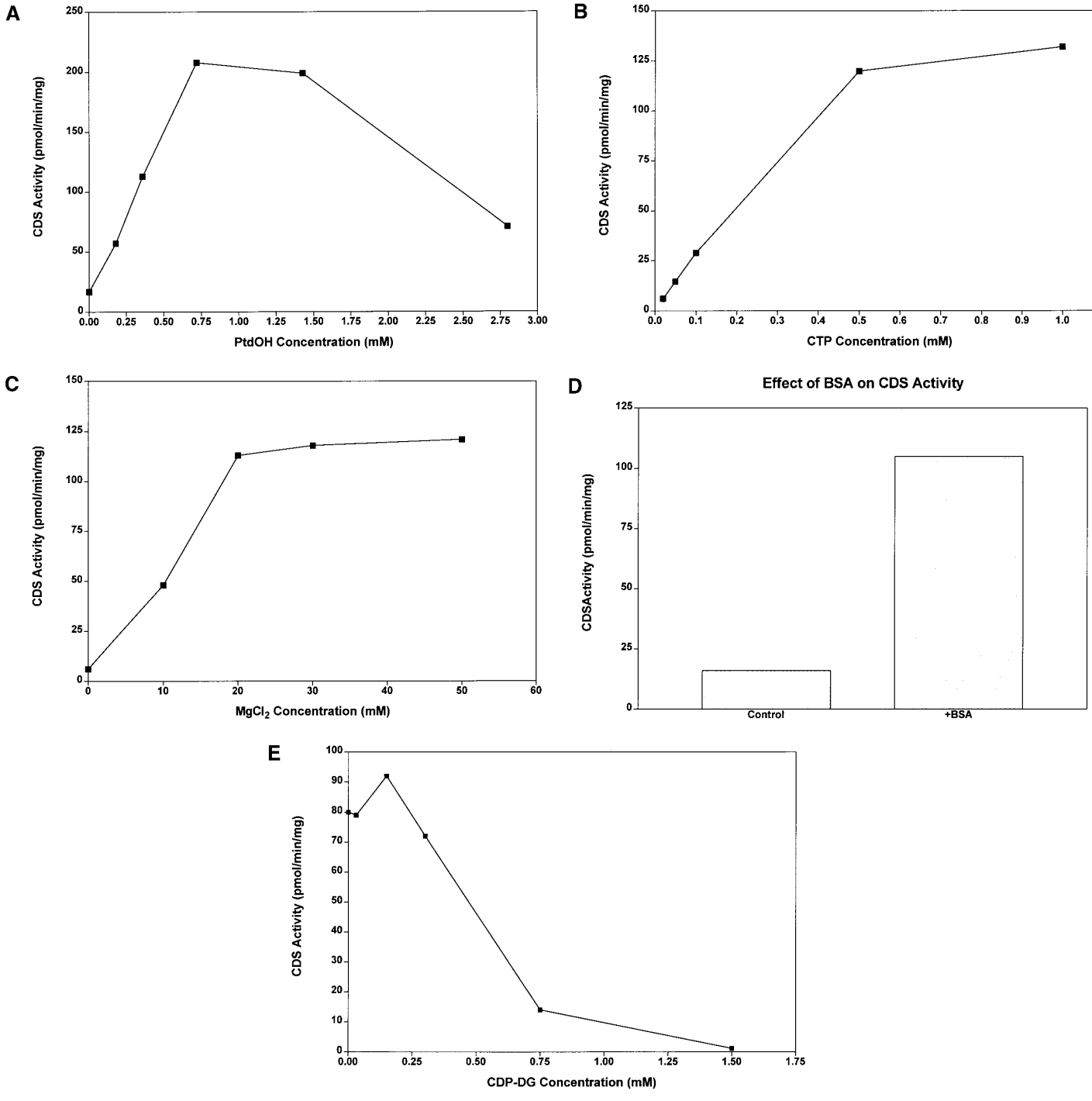

FIG. 2. Characterization of CDS Activity. **[A]** Dependence of CDS activity on the concentration of PtdOH. Assays were carried out as described in the text at a concentration of CTP of 1mM. Values represent the means of duplicate determinations. **[B]** Dependence of CDS activity on the concentration of CTP. The concentration of PtdOH was 1.4mM. Values represent the means of duplicate determinations. **[C]** Dependence of CDS activity on the concentration of $MgCl_2$. Concentrations of PtdOH and CTP were 1.4 and 1mM, respectively. Values represent the means of duplicate determinations. **[D]** Effect of BSA on CDS activity. Values represent the means of duplicate determinations. **[E]** Effect of CDP-DG on CDS activity. Concentrations of PtdOH and CTP were 1.4 and 1mM, respectively. Values represent the means of duplicate determinations.

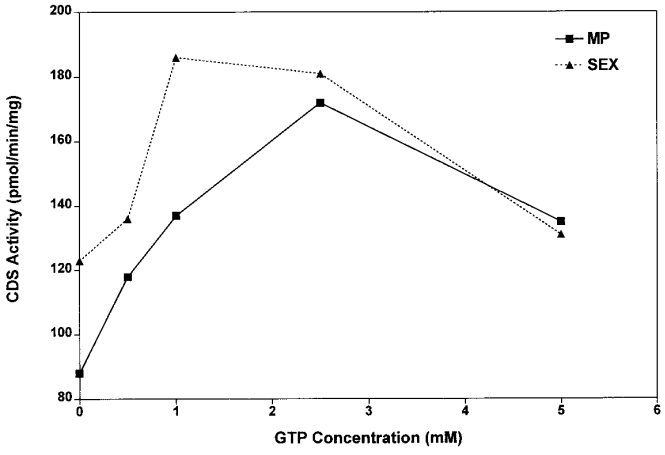

FIG. 3. Effect of GTP on CDS activity. The microsomal pellet (MP) and salt extract (SEX) activity were compared for sensitivity to GTP. Values shown represent the means of duplicate determinations.

phosphoinositide cycle (14-17). Taking this notion a step further, it has been shown in Drosophila that alterations in the level of expression of CDS effect signal transduction phenomena (18), and an increase in CDS expression in ECV304 endothelial cells results in an increased secretion of TNFα and IL-6 in response to stimulation with IL-1β (10).

Both the yeast and *E. coli* CDS have been purified (19-21). Purification of the mammalian enzyme, on the other hand, has remained elusive. This is most likely due to the fact that mammalian CDS, unlike that found in yeast and bacteria, is strongly inhibited by detergents. Even in the instance where the rat liver enzyme was extracted from the membrane by CHAPS, the activity was not stable, although addition of phospholipids partially restored the activity (5,6,22).

In the process of trying to extract active enzyme from rat liver microsomes, we tested a variety of detergents, including the non-ionic detergent, Hecameg, which we used successfully to purify mammalian phosphatidylinositol synthase (23),. In all cases there was a rapid loss of activity at concentrations of detergent required to solubilize membrane proteins.

To avoid the detergent-induced loss of activity, we extracted rat liver microsomes with 2M KCl. The resulting salt-extracted activity was stable indefinitely when stored at −72°C, and for at least 24 hours at 4°C. The characteristics of the extracted enzyme were very similar to those reported for the microsomal activity. The extracted enzyme was dependent on both CTP and PtdOH for activity, with Kms in the millimolar range, as originally reported by Carter and Kennedy for guinea pig liver (4). There was an absolute requirement for $MgCl_2$, as has been reported previously for both liver (4) and brain (24) preparations. The product, CDP-DG, inhibited the activity in a dose dependent fashion; however, neither $PtdInsP_2$ nor $InsP_3$ had any effect on activity (data not shown). Others have recently reported an inhibitory effect of $PtdInsP_2$ on CDS activity expressed in COS cells transfected with a full length cDNA for CDS (11). However, in this case, total lysates were used and detergent (Triton X-100, 0.3%) was present in the assay mix. It is possible that the conditions of our assay precluded adequate lipid solubilization; however, because of our previous experience with the inhibitory effects of detergents, we chose to avoid their use.

It has previously been reported that rat liver microsomal CDS activity was sensitive to GTP (6). In the present study, we were able to duplicate this finding, and to demonstrate that the same sensitivity applied to the salt-extracted CDS activity; however, the maximal degree of stimulation by GTP was always less in the extract than in the microsomal preparation. The mechanism involved in GTP stimulation of CDS activity remains to be determined.

In summary, we have succeeded in extracting CDS from microsomal membranes in a form that remains stable indefinitely at −72°C and for at least 24 hours at 4°C. This should greatly facilitate the further purification and characterization of the enzyme, including a more detailed assessment of factors which regulate its activity.

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