INCREASED MITOCHONDRIAL CTP: PHOSPHATIDIC ACID CYTIDYLTRANSFERASE IN THE 7777 HEPATOMA

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SUMMARY

CTP: phosphatidic acid cytidyltransferase is an important enzyme in the mitochondrial pathway for diphosphatidylglycerol (cardiolipin) synthesis. 7777 hepatoma mitochondria were found to have a 360% increase in CTP: phosphatidic acid cytidyltransferase as compared with normal liver mitochondria. 7777 hepatoma microsomes were significantly less active in CDP-diacylglycerol synthesis as compared with normal liver microsomes. In normal liver, the microsomal fraction is the most active in CDP-diacylglycerol synthesis but in the 7777 hepatoma the specific activity of CTP: phosphatidic acid cytidyltransferase was highest in the mito chondrial fraction. The results suggest that the subcellular localization of membrane-bound enzymes may be altered by malignant transformation and raise the possibility of abnormal membrane biogenesis in the 7777 hepatoma.

INTRODUCTION

The acidic phospholipids, phosphatidylglycerol and diphosphatidylglycerol (cardiolipin) are formed in liver mitochondria in reactions which require CDP-diacylglycerol as a substrate (1-3). CDP-diacylglycerol is nearly undetectable in mammalian liver. However, Thompson and MacDonald have recently isolated and characterized this compound in beef liver where it was found to be present in a concentration of approximately 5 to 17μMol per kg liver (4). The concentration of phosphatidic acid, which comprises about 0.7% of liver phospholipids, was estimated to be 780μMol per kg liver (4). Phosphatidylglycerol, which is derived from CDP-diacylglycerol and serves as a substrate for diphosphatidylglycerol synthesis represents about 0.6% of total liver phospholipid (5) and is present in a much higher concentration than CDP-diacylglycerol. Thus, the rate of de

novo synthesis of diphosphatidylglycerol in mitochondria is probably determined to a large degree by the availability of CDP-diacylglycerol. In normal liver, CTP: phosphatidic acid cytidyltransferase is primarily microsomal while mitochondria have only 10 to 20% of the activity present in microsomes (2,6).

The phospholipid content of 7777 hepatoma mitochondria relative to protein has recently been shown to be significantly increased (7). Since the activity of CTP: phosphatidic acid cytidyltransferase may be an important factor in the rate of mitochondrial diphosphatidylglycerol synthesis, we have studied this enzyme in the subcellular fractions from normal liver and the 7777 hepatoma.

MATERIALS AND METHODS

7777 hepatomas were maintained by subcutaneous passage in male rats of the Buffalo strain (Simonsen, Gilroy, Ca.). The rats were killed and the tumors excised, minced and washed in ice-cold 0.25 M sucrose containing 5 mM Tris/HCl (pH 7.4) and 2 mM EDTA; normal livers were obtained similarly from non-tumor bearing rats after an overnight fast. Subcellular fractions were prepared as described previously (7). Protein was measured by the method of Lowry et al (8); succinate dehydrogenase by the method of Green et al (9); rotenone-insensitive NADPH-cytochrome c reductase by the method of Sottacasa et al (10). CTP: phosphatidic acid cytidyltransferase was determined by the method of Hostetler and van den Bosch (2); details of the incubations are given in the legends.

Identification of the radioactive product obtained when (5-^{3}H)CTP was incubated with the various fractions was done as follows. 20 volumes of ice-cold chloroform/methanol (2/1 by volume) was added and the phases separated by the addition of 0.2 volumes of 0.01 N HCl (11). The lower (chloroform) phase was removed and washed once with cold upper phase and the resulting chloroform layer was removed and concentrated to a small volume with a nitrogen stream and analyzed by thin-layer chromatography. The following systems were used: system A, 0.25 mm silica gel G plates developed with chloroform/methanol/water/conc ammonia, (70/38/2/8 by volume); system B, 0.25 mm silica gel H plates prepared with 0.001 M Na_2CO_3 and developed with chloroform/methanol/glacial acetic acid/water (50/25/7/3 by volume), and system C, 0.25 mm silica gel G plates developed with chloroform/pyridine/formic acid (50/30/7 by volume). The radioactive lipid was located by scanning with a Panax thin-layer scanner (Panax Instruments, Redhill, Surrey, England). Phosphatidylcholine was isolated from egg yolks (12), converted to phosphatidic acid by the action of phospholipase D (Calbiochem, La Jolla, Ca) and purified by silicic acid column chromatography (13). CDP-diacylglycerol was synthesized from phosphatidic acid and CMP monophosphomorpholidate (Sigma, St. Louis, Mo.) and purified as previously described (14). The product was judged to be 99% pure by thin-layer chromatography. (5-^{3}H)CTP was obtained from New England Nuclear, Boston, Ma. Silica gels G and H and silica gel 60, extrapure (EM Reagents) were obtained from Brinkmann Instruments, Burlingame, Ca. Cytochrome c was obtained from Boehringer-Mannheim, San Francisco, Ca; NADPH and CTP from Sigma, St. Louis, Mo. Other chemicals were of analytic reagent grade; solvents were redistilled before use.

RESULTS AND DISCUSSION

The subcellular distribution of CTP: PA cytidyltransferase in a typical experiment is shown in Table I. The normal liver unwashed mitochondrial prep-

TABLE I. SUBCELLULAR LOCALIZATION OF CTP: PA CYTIDYLTRANSFERASE IN NORMAL RAT LIVER AND 7777 HEPATOMA.

FRACTION[b]	CTP: PA CYTIDYLTRANSFERASE[a]	
	NORMAL LIVER	7777 HEPATOMA
HOMOGENATE	281	181
UNWASHED MITOCHONDRIA	286	307
GRADIENT MITOCHONDRIA	78	324
MICROSOMES	436	174
SUPERNATANT	0	0

a picomoles CDP-diacylglycerol produced mg^{-1} min^{-1}

b the incubation mixture contained 50 mM Tris/HCl, pH 7.2; 2 mM (5-^{3}H)CTP, specific activity 9.7 mCi/mMole; 3 mg protein per ml; 1 mM phosphatidic acid; 40 mM $MgCl_2$ (added last) in a final volume of 0.333 ml. After a 20 min incubation at 37° duplicate 100 microliter aliquots were removed for analysis (2).

aration had considerable activity (286 picomoles mg^{-1} min^{-1}) due in large part to microsomal contamination since the specific activity of the gradient-purified mitochondria was only 78 picomoles mg^{-1} min^{-1}. The normal liver microsomal fraction was the most active in CDP-diacylglycerol formation with a specific activity of 436 picomoles mg^{-1} min^{-1}. However, the subcellular localization of this enzyme in the 7777 hepatoma was different in that the mitochondrial fraction had the highest CTP: PA cytidyltransferase specific activity. The unwashed mitochondrial fraction had a specific activity of 307 picomoles mg^{-1} min^{-1}. Further purification of the crude mitochondrial fraction by gradient centrifugation resulted in an increase in the specific activity to 324 picomoles mg^{-1} min^{-1} which would be expected with an enzyme having greater activity in mitochondria than in other subcellular fractions. The 7777 hepatoma microsomal fraction CTP: PA cytidyltransferase activity was 174 picomoles mg^{-1} min^{-1} which is considerably lower than that of normal liver microsomes (2). No CTP: PA cytidyltransferase activity was found in the supernatant fraction of either normal liver or hepatoma.

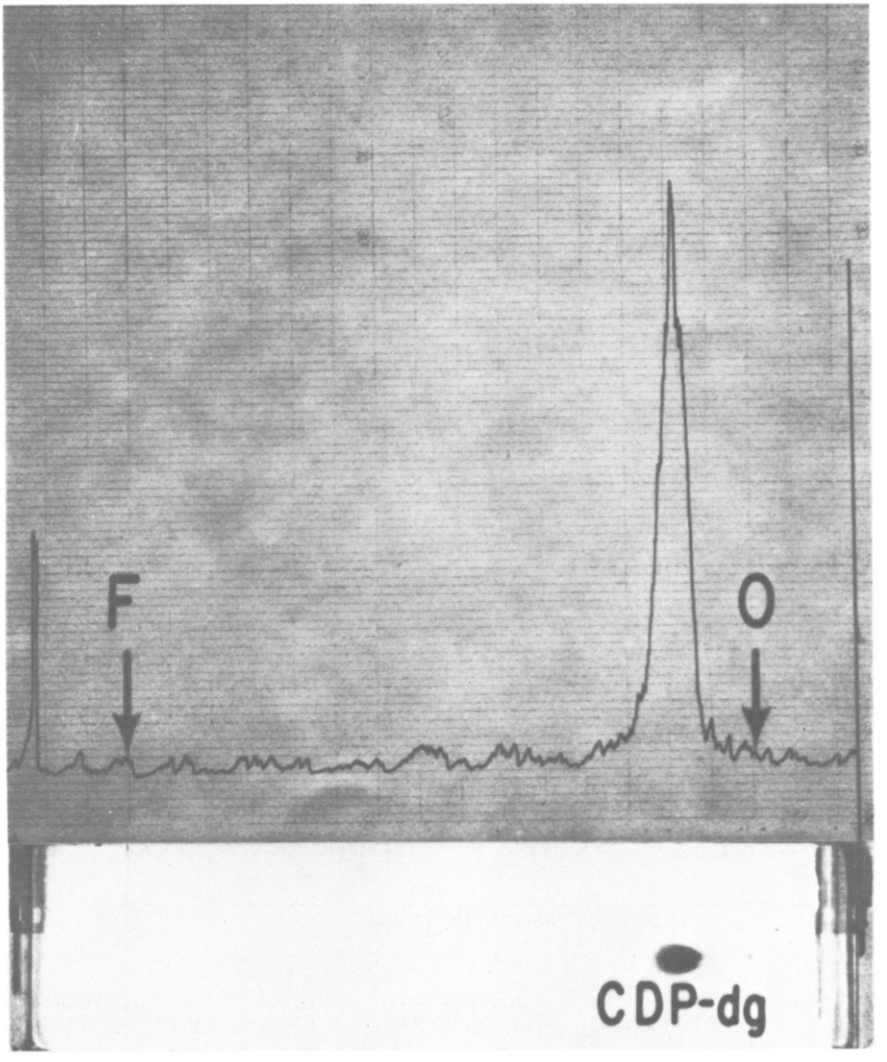

Figure 1. Thin-layer chromatography of reference CDP-diacylglycerol and the ^{3}H-labeled lipid formed by 7777 hepatoma mitochondria. The lipids were extracted as described in Methods and chromatographed in system A.

Since the results in Table I were obtained with an assay which employs trichloroacetic acid precipitation of the ^{3}H-labeled lipid product on filter papers (2,15), further steps were taken to identify the compound formed as CDP-diacylglycerol. Figure 1 shows a photograph of a thin-layer chromatogram and scan of reference CDP-diacylglycerol and the ^{3}H-labeled lipid product obtained when 7777 mitochondria were incubated with (5-^{3}H)CTP and phosphatidic acid (PA). It is

readily apparent that the Rf of the radioactive lipid corresponds with reference CDP-diacylglycerol in this system. The radioactive lipid also cochromatographed with pure CDP-diacylglycerol in system B (Rf 0.39 versus 0.38) and in system C (Rf 0.02 vs. 0.02). The lipid products formed from (5-^{3}H)CTP and PA by hepatoma microsomes and normal liver microsomes and mitochondria also cochromatographed with reference CDP-diacylglycerol in all three chromatography systems, confirming the identity of the respective radioactive products.

The properties of CDP-diacylglycerol synthesis were investigated further in fractions from the 7777 hepatoma and normal liver. As shown in Table II, the reaction was dependent on the concentrations of both Mg^{2+} and PA. No major differences were apparent between normal liver and hepatoma fractions in the absence of Mg^{2+} or PA. At low concentrations of PA (0.1 mM) and Mg^{2+} (5 mM), increased rates of CDP-diacylglycerol synthesis were observed in 7777 hepatoma mitochondria; hepatoma microsomes were less active as compared with the respective fract-

TABLE II. PROPERTIES OF CDP-DIACYLGLYCEROL BIOSYNTHESIS IN SUBCELLULAR MEMBRANE FRACTIONS FROM NORMAL RAT LIVER AND 7777 HEPATOMA.

	CTP: PA CYTIDYLTRANSFERASE [a]			
ADDITIONS[b]	NORMAL LIVER MITOCHONDRIA	7777 HEPATOMA MITOCHONDRIA	NORMAL LIVER MICROSOMES	7777 HEPATOMA MICROSOMES
COMPLETE	87	333	486	178
NO Mg^{2+}	22	18	10	15
5 mM Mg^{2+}	75	278	344	72
NO PA	11	10	45	29
0.1 mM PA	30	151	65	45
70° x 10 MIN	0.8	0	17	27

a- picomoles CDP-diacylglycerol produced mg^{-1} min^{-1}

b- additions to the complete mixture were the same as shown in Table I. Incubations were done at 37° for 20 min with changes as noted.

ions from normal liver. Heating the protein fractions at 70^0 for 10 min before starting the reaction by adding the substrates abolished the activity in both normal and tumor mitochondria. However, some residual activity was observed in both microsomal preparations. The complete incubation mixtures again showed that CTP: PA cytidyltransferase was increased in tumor mitochondria and decreased in tumor microsomes as compared with the respective normal liver fractions.

The purity of several different microsomal and gradient purified mitochondrial fractions was assessed by measurement of succinate dehydrogenase and rotenone-insensitive NADPH-cytochrome c reductase. The results are shown in Table III. Microsomal contamination of the mitochondrial fraction was estimated at 3% for normal liver and 5% for the 7777 hepatoma, respectively. Mitochondrial contamination of the microsomes was 6% for both normal liver and the 7777 hepatoma. Table III also shows the activity of CTP: PA cytidyltransferase in mitochondria in three separate experiments; it was found to be increased by 360% in the 7777 hepatoma mitochondria and decreased by 62% in tumor microsomes as compared with the respective fractions from normal liver. In the 7777 hepatoma, mitochondria were more active in CDP-diacylglycerol synthesis than the microsomes, 298 picomoles $mg^{-1}min^{-1}$ versus 168 picomoles mg^{-1} min^{-1}. However, in normal rat liver

TABLE III. ACTIVITY OF MARKER ENZYMES AND CTP: PA CYTIDYLTRANSFERASE IN SUBCELLULAR FRACTIONS FROM NORMAL RAT LIVER AND 7777 HEPATOMA.

SUBCELLULAR FRACTION	SUCCINATE DEHYDROGENASE *a*	ROTENONE-INSENSITIVE NADPH-CYT C REDUCTASE *a*	CTP: PA CYTIDYLTRANSFERASE *b*
LIVER MITOCHONDRIA (3)	16.5 ± 0.9	1.2 ± 1.1	83.2 ± 10.7
LIVER MICROSOMES (3)	0.9 ± 0.4	47.3 ± 3.9	441.0 ± 6.8
HEPATOMA MITO (3)	12.6 ± 1.5	0.5 ± 0.2	298.0 ± 45
HEPATOMA MICRO (3)	0.8 ± 0.5	9.6 ± 0.8	168.0 ± 12

a- nanomoles mg^{-1} min^{-1} ± 1 std. deviation

b- picomoles mg^{-1} min^{-1} ± 1 std. deviation; incubation conditions as in Table I

the situation is opposite. The microsomal fraction had the highest specific activity, 441 picomoles $mg^{-1}min^{-1}$, while mitochondrial activity was 83 picomoles mg^{-1} min^{-1}. The values for normal liver subcellular fractions are in good agreement with other published results (2).

The cause of the increased 7777 hepatoma mitochondrial CTP: PA cytidyltransferase activity is not known but it may be a result of malignant transformation. One possible explanation for this finding would be abnormal biogenesis of mitochondria in the 7777 hepatoma. The increased activity of CTP: PA cytidyltransferase in the 7777 hepatoma would be expected to lead to increased synthesis of CDP-diacylglycerol, an important determinant of the rate of mitochondrial diphosphatidylglycerol synthesis, and might explain the increased levels of diphosphatidylglycerol which have been reported (7). Further studies are necessary to examine the various possible explanations for the abnormal subcellular localization of CTP: PA cytidyltransferase in the 7777 hepatoma.

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REFERENCES

1. Hostetler, K.Y., van den Bosch, H. and van Deenen, L.L.M. (1971) Biochim. Biophys. Acta, 239, 113-119.
2. Hostetler, K.Y., van den Bosch, H. (1972) Biochim. Biophys. Acta, 260, 380-386.
3. Hostetler, K.Y., van den Bosch, H. and van Deenen, L.L.M. (1972) Biochim. Biophys. Acta, 260, 507-513.
4. Thompson, W. and MacDonald, G. (1975) J. Biol. Chem, 250, 6779-6785.
5. Poorthuis, B.J.H.M., Yazaki, P.J. and Hostetler, K.Y. (1976) J. Lipid Res, in press.
6. Carter, J.R. and Kennedy, E.P. (1966) J. Lipid Res, 7, 678-683.
7. Hostetler, K.Y., Zenner, B.D., and Morris, H.P. (1976) Biochim. Biophys. Acta, in press.
8. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem, 193, 265-275.
9. Green, D.E., Mii, S. and Kohout, P.M. (1955) J. Biol. Chem, 217, 551-567.

10. Sottacasa, G.L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1967) J. Cell Biol, 32, 415-438.
11. Folch, J., Leas, M. and Sloane Stanley, G.H. (1957) J. Biol. Chem, 217, 497-509.
12. Singleton, W.S., Gray, M.S., Brown, M.L. and White, J.L. (1965) J. Am. Oil Chem. Soc, 42, 53-56.
13. Long, C., Odavic, R. and Sargent, E.J. (1967) Biochem. J. 102, 216-220.
14. Hostetler, K.Y., Galesloot, J.M., Boer, P. and van den Bosch, H. (1975) Biochim. Biophys. Acta, 380, 382-389.
15. Goldfine, H. (1966) J. Lipid Res, 7, 146-149.