BBA 56026

SUBCELLULAR AND SUBMITOCHONDRIAL LOCALIZATION OF THE BIOSYNTHESIS OF CARDIOLIPIN AND RELATED PHOSPHOLIPIDS IN RAT LIVER

K. Y. HOSTETLER AND H. VAN DEN BOSCH
Laboratory of Biochemistry, State University of Utrecht, Vondellaan 26, Utrecht (Netherlands)
(Received October 26th, 1971)

SUMMARY

1. Intact mitochondria, inner and outer mitochondrial membranes and microsomes were isolated from rat liver and their purity determined with specific marker enzymes.

2. Diphosphatidylglycerol synthesis was found exclusively in the inner mitochondrial membrane. Phosphatidylglycerol was predominantly synthesized in the inner membrane although a small contribution was noted in the outer membrane. CDP and dCDP diglyceride synthesis occurred primarily in the microsomal fraction.

3. Diphosphatidylglycerol synthesis from phosphatidylglycerol was shown to require CDP diglyceride or dCDP diglyceride and a divalent cation (either Mg^{2+}, Mn^{2+} or Co^{2+}). The reaction was strongly inhibited by nonionic detergents such as Triton X-100

INTRODUCTION

Although mitochondria apparently do not participate in the biosynthesis of the major phospholipids, *e.g.* phosphatidylcholine and phosphatidylethanolamine[1-3], they do have the capacity to synthesize phosphatidic acid[4-6], CDP diglyceride[7], dCDP diglyceride[8] and phosphatidylglycerol[9-11]. A recent report from this laboratory described diphosphatidylglycerol (cardiolipin) biosynthesis in mitochondrial preparations from normal and germ-free rat liver[9].

Phosphatidic acid is synthesized predominantly in the outer mitochondrial membrane[4-6], but the submitochondrial location of CDP diglyceride, phosphatidylglycerol and diphosphatidylglycerol synthesis has not yet been reported. This paper reports the subcellular and submitochondrial localization of the synthesis of diphosphatidylglycerol and its precursor, phosphatidylglycerol. The distribution of CDP and dCDP diglyceride biosynthesis has also been determined. Some properties of the reaction leading to diphosphatidylglycerol are described.

METHODS

Preparation of subcellular fractions

Subcellular fractions were prepared from the livers of fasted male rats essentially according to the procedure described by SARZALA *et al.*[5] except that the rats were not injected with Triton WR 1339. Inner and outer mitochondrial membranes were isolated by the method of SCHNAITMAN *et al.*[12]. This procedure yields an outer membrane fraction, an interstitial soluble protein fraction and an inner membrane fraction which still contains the matrix. No attempts were made to separate matrix proteins from the inner membrane. After determination of protein by the method of LOWRY *et al.*[13], the fractions were suspended in a solution of 0.25 M sucrose–5 mM Tris (pH 7.5) and stored at −18° until use.

Marker enzymes

Glucose-6-phosphatase (EC 3.1.3.9.) and rotenone-insensitive NADPH–cytochrome *c* reductase (EC 1.6.2.3) were used as microsomal marker enzymes and were assayed as described in refs. 14 and 15. The inner mitochondrial membrane markers, succinate dehydrogenase (EC 1.3.99.1) and cytochrome oxidase (EC 1.9.3.1) were assayed as described in refs. 16 and 12. Monoamine oxidase (EC 1.4.3.4) was determined spectrophotometrically using kynuramine dihydrobromide as substrate according to the method of WEISSBACH *et al.*[17]. Rotenone-insensitive NADH–cytochrome *c* reductase (EC 1.6.2.1) was assayed as described by SOTTACASA *et al.*[15].

Radioactive substrates

sn-[2-^{3}H]-Glycerol-3-phosphate was prepared from [2-^{3}H]glycerol by the method of CHANG AND KENNEDY[18]. CDP and deoxy CDP diglycerides were prepared from phosphatidic acid (source: egg lecithin) as described by AGRANOFF AND SUOMI[19] and modified by PROTTEY AND HAWTHORNE[20].

Phosphatidyl[2-^{3}H]glycerol was obtained biosynthetically under the following conditions: the incubation mixture contained 50 mg mitochondrial protein; 50 mM Tris (pH 7.5); 20 mM β-mercaptoethanol; $1.5 \cdot 10^{-4}$ M *sn*-[2-^{3}H]glycerol 3-phosphate (spec. act. 300 mC/mmole); $8 \cdot 10^{-5}$ M CDP diglyceride in a final volume of 5.0 ml. Incubation was for 2 h at 37°. The total lipids were extracted by the method of BLIGH AND DYER[21] and applied to a 1.6 cm × 10 cm column of silica gel (Merck Kieselgel ASTM 75–360 mesh) which was eluted with 4%, 12% and 25% fractions of methanol in chloroform. The crude phosphatidyl[2-^{3}H]glycerol was generally recovered in the 25% fraction. Final purification was achieved by thin-layer chromatography using silica gel H impregnated with 0.1 M $NaHCO_3$ and developed with chloroform–methanol–1 M ammonia (80:36:2, by vol.) (System I). Radioactivity was located using a Panax thin-layer chromatography scanner (Panax Equipment Ltd., Redhill, Surrey, England); the area corresponding to phosphatidyl[^{3}H]glycerol was removed and eluted with three successive 3-ml volumes of chloroform–methanol (2:1, by vol.). The final product gave a single radioactive peak in thin-layer chromatography Systems I and II which co-chromatographed with reference phosphatidylglycerol and was not contaminated with unlabeled phospholipids as judged by development with iodine vapors and phosphorous spray[22]. This product gives radioactive glycerol when treated with phospholipase D[23] and on alkaline hydrolysis gives glycerylphosphoryl glycerol[9],

thus establishing its identity as phosphatidylglycerol. Phosphorus was determined by the method of AMES AND DUBIN[24].

Assays of phospholipid synthesis

Diphosphatidylglycerol synthesis. Phosphatidyl[2-^{3}H]glycerol and CDP diglyceride were prepared for incubation by brief sonication in distilled water at 0° using a Branson Sonifier (Branson Sonic Power, Danbury, Conn., U.S.A.) with microtip. Incubation conditions are described in the respective legends. Lipid extractions were carried out by the method of BLIGH AND DYER[21]. The total lipid extract was applied to thin-layer chromatography plates of silica gel H impregnated with 0.4 M boric acid and developed with chloroform–methanol–water–conc. ammonia (70:30:3:2, by vol.) (System II). Radioactive diphosphatidylglycerol was located with a thin-layer chromatography scanner followed by development of diphosphatidylglycerol reference spots with iodine vapors. This material has previously been shown to give bis (glycerylphosphoryl)- glycerol on mild alkaline hydrolysis[9]. The areas representing radioactive diphosphatidylglycerol were placed in scintillation vials containing toluene (with 0.5% PPO and 0.03% POPOP)–Triton X-100–water (2:1:0.1, by vol.). Measurements of radioactivity were made using a Packard Tri Carb liquid scintillation counter with external standard quench corrections.

Phosphatidylglycerol synthesis. Phosphatidylglycerol formation was assayed as described by TER SCHEGGET *et al.*[8].

CDP and dCDP diglyceride synthesis. CDP and dCDP diglycerides were assayed as follows: the incubation mixture contained 50 mM Tris (pH 7.2), 1 mg of protein as indicated, 3 mM [U-^{14}C]CTP (spec. act. $1.3 \cdot 10^6$ disint./min per μmole) or 3 mM-[5-^{3}H]dCTP (spec. act. $7.8 \cdot 10^6$ disint./min per μmole) and 250 μg phosphatidic acid. Either 8 mM or 40 mM $MgCl_2$ was added last. The final volume was 0.33 ml and the incubation was for 20 min at 37°. Formation of radioactive liponucleotides was measured by the filter disc assay of GOLDFINE[25] modified as follows: the filter discs were passed through the following four trichloroacetic acid solutions at 0°: 10%, 5%, 1.5% and 1.5% instead of the system described by GOLDFINE[25] which employs water as the fourth solution. Labeled CDP and dCDP diglyceride were isolated from the remaining mixtures and characterized by thin-layer chromatography. Single radioactive peaks were observed which co-chromatographed with synthetic CDP diglyceride and dCDP diglyceride, respectively, in two solvent systems as previously described by TER SCHEGGET *et al.*[8].

Chemicals

All radioactive compounds were purchased from the Radiochemical Centre, Amersham, England. Cytidine 5'-monophosphomorpholidate and deoxycytidine 5'-monophosphomorpholidate and adenosine triphosphate were obtained from Sigma Co., St. Louis, Mo., U.S.A. Glycerol kinase was purchased from Boehringer, Mannheim, Germany. All other reagents were obtained from commercial sources and were of Analytic Reagent grade.

RESULTS

Table I shows the specific activities of several marker enzymes for mitochondria, microsomes and submitochondrial fractions. Based on rotenone-insensitive NADPH–

TABLE I

SPECIFIC ACTIVITIES OF MARKER ENZYMES

The results are expressed as nmoles substrate converted per mg protein per min. Monoamine oxidase activity is given as change in $A_{300\ nm}$ per mg per min.

Subcellular fraction	*Monoamine oxidase*	*Succinate dehydrogenase*	*Cytochrome oxidase*	*Rotenone-insensitive NADH–cyt. c reductase*	*Rotenone-insensitive NADPH–cyt. c reductase*	*Glucose-6-phosphatase*
Intact mitochondria	0.06	24.1	n.d.*	n.d.	1.5	n.d.
Mitochondria after digitonin	0.05	27.7	70.5	454	2.3	8.8
Inner membrane	0.02	30.9	92.0	113	1.5	0.0
Outer membrane	0.54	5.2	20.8	3215	5.3	33.6
Interstitial soluble protein	0.04	1.0	0.0	242	0.7	10.4
Microsomes	0.01	0.5	0.0	2060	36.4	276

* n.d. = not determined.

cytochrome *c* reductase and glucose-6-phosphatase specific activities, a maximum contamination of 3% was calculated for microsomal contamination of the mitochondrial preparation, whereas the microsomes were about 2% contaminated with mitochondrial outer membranes based on monoamine oxidase. The inner membrane markers succinate dehydrogenase and cytochrome oxidase indicated that the microsomes are no more than 2% contaminated with inner membranes. Mitochondrial inner membranes were estimated to be less that 4% contaminated with either outer membranes or microsomes. Outer membrane contamination with inner membrane was found to be 17% based on succinate dehydrogenase and 23% based on cytochrome oxidase. Microsomal contamination of the outer membrane was estimated to be 12%.

Diphosphatidylglycerol synthesis was found exclusively in the mitochondrial inner membrane fraction (Table II). The diphosphatidylglycerol synthesis observed in the outer membrane and microsomal fractions could be completely accounted for by contamination of these fractions with inner membranes. Phosphatidylglycerol synthesis was predominantly localized in the inner membrane fraction but a small contribution was also noted in mitochondrial outer membranes. Finally, the interstitial soluble protein fraction was not active in diphosphatidylglycerol or phosphatidylglycerol synthesis.

When CDP diglyceride and dCDP diglyceride was assayed in the presence of

TABLE II

LOCALIZATION OF PHOSPHATIDYLGLYCEROL AND DIPHOSPHATIDYLGLYCEROL SYNTHESIS

Data given in pmoles per mg protein per min. Diphosphatidylglycerol assay mixture contained $2 \cdot 10^{-5}$ M CDP diglyceride; 50 mM Tris (pH 7.5); $7 \cdot 10^{-6}$ M phosphatidyl[2-^{3}H]glycerol, spec. act. 240 μC/μmole; 1.07 mg protein; 2 mM EDTA; 10 mM $MgCl_2$ was added last. Final volume was 0.25 ml; incubated for 1 h at 37°.

Subcellular fraction	*Phosphatidylglycerol*	*Diphosphatidylglycerol*
Intact mitochondria	39.8	2.950
Mitochondria after digitonin	48.6	0.715*
Inner membrane	63.1	2.830
Outer membrane	17.7	0.210
Interstitial soluble proteins	1.0	0
Microsomes	15.7	0.020

* This low value is apparently due to the presence of digitonin in this fraction, see also Fig. 1.

8 mM Mg^{2+} (Table III), the microsomes were found to be several fold more active than intact mitochondria. Most of the mitochondrial activity was found in the inner membrane and this could not be due to the presence of 4% microsomal contamination whereas the activity observed in the outer membrane fraction could be completely accounted for on the basis of a microsomal contamination of 12%. While we cannot

TABLE III

LOCALIZATION OF CYTIDINE LIPONUCLEOTIDE SYNTHESIS

Assays were carried out as described in METHODS. Data are given in pmoles/mg per min.

Subcellular fraction	*8 mM Mg^{2+}*		*40 mM Mg^{2+}*	
	CDP diglyceride	*dCDP diglyceride*	*CDP diglyceride*	*dCDP diglyceride*
Intact mitochondria	39.8	110.0	54.0	80.0
Mitochondria after digitonin	19.3	46.6	—	—
Inner membrane	34.7	92.5	40.4	68.0
Outer membrane	12.3	19.7	42.3	75.0
Interstitial soluble protein	0	1.9	—	—
Microsomes	140.0	250.0	368.0	500.0

completely exclude the presence of CTP:phosphatidic acid cytidyltransferase in the outer mitochondrial membrane, it is quite clear that the enzyme is predominantly located in the inner membrane. The assay was repeated using a 40 mM Mg^{2+} concentration, since the results of VORBECK AND MARTIN[7] using 10 mM Mg^{2+} were at variance with the data of CARTER AND KENNEDY[26] who used higher Mg^{2+} concentrations. With respect to CDP diglyceride formation, the activity of intact mitochondria increased moderately while a several-fold increase was noted in microsomes; mitochondrial formation of dCDP diglyceride actually decreased at high Mg^{2+} concentration. With high Mg^{2+} concentration, the activity of the outer membrane fraction was about equal to the inner membrane fraction. This can be accounted for by the presence of a 12% microsomal contamination in the outer membrane fraction.

TABLE IV

EFFECT OF CYTIDINE LIPONUCLEOTIDES, Mg^{2+} AND HEATING ON MITOCHONDRIAL DIPHOSPHATIDYLGLYCEROL FORMATION

Data expressed in pmoles/mg protein per h. Cytidine liponucleotides were present in concentrations indicated. Other components of the assay mixture were present as indicated in Table II.

Condition	*Diphosphatidylglycerol formation*
70° × 10 min/CDP diglyceride ($8 \cdot 10^{-6}$ M)	< 4
Mg^{2+} omitted/CDP diglyceride ($8 \cdot 10^{-6}$ M)	< 5
No added CDP diglyceride	< 4
CDP diglyceride ($5 \cdot 10^{-7}$ M)	30
CDP diglyceride ($5 \cdot 10^{-6}$ M)	90
dCDP diglyceride ($5 \cdot 10^{-7}$ M)	23
dCDP diglyceride ($5 \cdot 10^{-6}$ M)	53

Table IV demonstrates that diphosphatidylglycerol formation requires Mg^{2+} and either CDP or dCDP diglyceride. It was possible to replace Mg^{2+} with Mn^{2+} or Co^{2+} but not with Ca^{2+}. Heating of the mitochondrial protein at 70° for 10 min before the addition of substrates and Mg^{2+} results in a nearly complete inhibition of the reaction.

The formation of diphosphatidylglycerol is strongly inhibited by nonionic detergents as shown in Fig. 1. Some of these detergents have been routinely used in assays of phosphatidylglycerol synthesis[10,11].

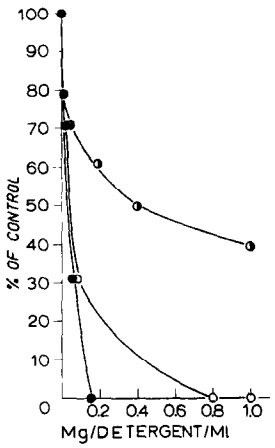

Fig. 1: Inhibition of diphosphatidylglycerol synthesis by detergents. The incubation mixture contained the detergent in concentrations as indicated; 50 mM Tris (pH 7.5); $8 \cdot 10^{-5}$ M CDP diglyceride; $6 \cdot 10^{-6}$ M phosphatidyl[2-^{3}H]glycerol (spec. act. 110 μC/μmole); 20 mM β-mercaptoethanol; 2 mM EDTA; 1.25 mg mitochondrial protein; 10 mM $MgCl_2$ was the last addition. Final volume was 0.250 ml. Incubation was for 1 h at 37°. ◑, Triton WR-1339; ○, Triton X-100; ●, Tween 20.

DISCUSSION

In rat and quinea pig liver, diphosphatidylglycerol is found almost exclusively in the mitochondrion, specifically in the inner membrane[27]. This report indicates that the inner membrane of the mitochondrion is the exclusive site of diphosphatidylglycerol synthesis as might be expected. The reaction utilizes phosphatidylglycerol and requires a divalent cation (Mg^{2+}, Mn^{2+} or Co^{2+}) and either CDP or dCDP diglyceride. The reaction is strongly inhibited by nonionic detergents such as Triton X-100. This may explain in part why other investigators did not observe diphosphatidylglycerol synthesis during their studies of phosphatidylglycerol synthesis in mitochondria[10,11]. However, more recent studies by DAVIDSON AND STANACEV[28,29] have shown conversion of labeled *sn*-glycerol 3-phosphate into diphosphatidylglycerol by guinea pig liver mitochondria in the presence of acyl-CoA and CDP diglyceride generating systems, but not in the presence of exogenous CDP diglyceride. Under these conditions, no incorporation of labeled *sn*-glycerol 3-phosphate into diphosphatidylglycerol was observed in the microsomal fraction in agreement with our findings using labeled phosphatidylglycerol (Table II).

In addition to diphosphatidylglycerol, phosphatidylglycerol is also synthesized primarily in the inner membrane of the mitochondrion although some synthesis occurs in the outer membrane. CDP diglyceride and deoxy CDP diglyceride synthesis is most active in the microsomal fraction; in the mitochondria, cytidine liponucleotide biosynthesis occurs principally in the inner membrane fraction. These biosynthetic capabilities contrast with the apparent inability of the mitochondrion to synthesize

the major phospholipid components, phosphatidylcholine and phosphatidylethanolamine[1-3].

ACKNOWLEDGEMENTS

This work was supported by funds from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.). K. Y. H. is a Postdoctoral fellow of the Department of Medicine, Case Western Reserve University, Cleveland, Ohio, U.S.A. (U.S. Public Health Service Grant Number AM-1005). The authors wish to thank Prof. Dr. L. L. M. van Deenen for his encouragement during this work and Mr. A. J. Aarsman for his excellent technical assistance.

REFERENCES

1 K. W. A. Wirtz and D. B. Zilversmit, *J. Biol. Chem.*, 243 (1968) 3596.
2 W. C. McMurray, R. M. C. Dawson, *Biochem. J.*, 112 (1969) 91.
3 M. T. Sauner and L. Lévy, *J. Lipid Res.*, 12 (1971) 71.
4 J. Zborowski and L. Wojtczak, *Biochim. Biophys. Acta*, 187 (1969) 73.
5 M. G. Sarzala, L. M. G. van Golde, B. de Kruyff and L. L. M. van Deenen, *Biochim. Biophys. Acta*, 202 (1970) 106.
6 E. H. Shepherd and G. Hübscher, *Biochem. J.*, 113 (1969) 429.
7 M. L. Vorbeck and A. P. Martin, *Biochem. Biophys. Res. Commun.* 40 (1970) 901.
8 J. Ter Schegget, H. van den Bosch, M. A. van Baak, K. Y. Hostetler and P. Borst, *Biochim. Biophys. Acta*, 239 (1971) 234.
9 K. Y. Hostetler, H. van den Bosch and L. L. M. van Deenen , *Biochim. Biophys. Acta*, 239 (1971) 113.
10 J. Y. Kiyasu, R. A. Pieringer, H. Paulus and E. P. Kennedy, *J. Biol. Chem.*, 238 (1963) 2293.
11 J. B. Davidson and N. Z. Stanacev, *Can. J. Biochem.*, 48 (1970) 633.
12 C. Schnaitman, V. G. Erwin and J. W. Greenwalt *J. Cell Biol.*, 32 (1967) 719.
13 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
14 M. A. Swanson, *J. Biol. Chem.*, 184 (1950) 647.
15 G. L. Sottacasa, B. Kuylenstierna, L. Ernster and A. Bergstrand, *J. Cell Biol.*, 32 (1967) 415.
16 D. E. Green, S. Mii and P. M. Kohout, *J. Biol. Chem.*, 217 (1955) 551.
17 H. Weissbach, T. E. Smith, J. W. Daly, B. Witkop and S. Udenfriend, *J. Biol. Chem.*, 235 (1960) 1160.
18 Y. Y. Chang and E. P. Kennedy, *J. Lipid Res.*, 8 (1967) 447.
19 B. W. Agranoff and W. D. Suomi, *Biochem. Prep.*, 10 (1963) 46.
20 C. Prottey and J. N. Hawthorne, *Biochem. J.*, 105 (1967) 379.
21 E. G. Bligh and W. J. Dyer, *Can. J. Biochem.*, 37 (1959) 911.
22 J. C. Dittmer and R. L. Lester, *J. Lipid Res.*, 5 (1964) 126.
23 E. J. Victoria, L. M. G. van Golde, K. Y. Hostetler, G. L. Scherphof and L. L. M. van Deenen, *Biochim. Biophys. Acta*, 239 (1971) 443.
24 B. N. Ames and D. T. Dubin, *J. Biol. Chem.*, 235 (1960) 769.
25 H. Goldfine, *J. Lipid Res.*, 7 (1966) 146.
26 J. R. Carter and E. P. Kennedy, *J. Lipid Res.*, 7 (1966) 678.
27 D. F. Parsons, G. R. Williams, W. Thompson, D. F. Wilson and B. Chance, in E. Quagliariello, S. Papa, E. C. Slater and J. M. Tager, *Mitochondrial Structure and Compartmentation*, Adriatica Editrice, 1967, p. 29.
28 J. B. Davidson and N. Z. Stanacev, *Biochem. Biophys. Res. Commun.*, 42 (1971) 1191.
29 J. B. Davidson and N. Z. Stanacev, *Can. J. Biochem.*, 49 (1971) 1117.