BIOSYNTHESIS OF CARDIOLIPIN IN MITOCHONDRIA ISOLATED FROM GUINEA PIG LIVER

J.B. Davidson and N.Z. Stanacev

Department of Pathological Chemistry, Banting Institute
Faculty of Medicine, University of Toronto, Toronto 181, Ontario, Canada

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SUMMARY

Mitochondria and microsomes isolated from guinea pig liver were investigated for their ability to catalyze the biosynthesis of cardiolipin in a system containing L-glycero-3-phosphate-2-^{3}H and exogenously added CDP-D-diglyceride, and in a system containing L-glycero-3-phosphate-2-^{3}H and generating endogenously CDP-D-diglyceride. Only with mitochondria and with the system generating CDP-D-diglyceride was the biosynthesis of cardiolipin detected. These results in conjunction with previously described results from this and other laboratories have established mitochondrial capability for the biosynthesis of all polyglycerophosphatides (phosphatidylglycerophosphate, phosphatidylglycerol and cardiolipin).

INTRODUCTION

The formation of cardiolipin, a characteristic phospholipid of mitochondria (1) and mitochondrial inner membrane (2), has been successfully studied only in bacteria (3) thus far. In our attempt to study the biosynthesis of polyglycerophosphatides in mammalian tissue, we have examined a system containing L-glycero-3-phosphate-2-^{3}H and exogenously added CDP-D-diglyceride as substrates, and sheep brain and beef heart subcellular particles as the sources of enzymes, anticipating that the biosynthesis of cardiolipin would take place according to the mechanism represented by reaction scheme (I).

L-Glycero-3-phosphate + CDP-D-diglyceride → Phosphatidylglycerophosphate....(a)

Phosphatidylglycerophosphate → Phosphatidylglycerol....(b)

Phosphatidylglycerol + CDP-D-diglyceride → Cardiolipin....(c)

L-Glycero-3-phosphate + 2 CDP-D-diglyceride → Cardiolipin....(I)

We have found, however, that the formation of compounds identified as

phosphatidylglycerophosphate and phosphatidylglycerol occurred without the formation of a detectable amount of cardiolipin (4,5).

We wish to report now the enzymatic synthesis of cardiolipin, apparently according to reaction scheme (I), in a system containing L-glycero-3-phosphate-2-^{3}H and endogenously generating CDP-D-diglyceride catalyzed by guinea pig liver mitochondria.

MATERIALS AND METHODS

L-Glycero-3-phosphate-2-^{3}H and CDP-D-diglyceride were of the origin and purity described (5). All other chemicals and reagents were of commercial origin and were of the highest purity available.

Mitochondria and microsomes were prepared from guinea pig liver according to methods described (6). "Purified mitochondria" were obtained by centrifugation in a discontinuous gradient of sucrose (7). Microsomal contamination of purified mitochondria was 5-8%, as judged by the determination of glucose-6-phosphatase (8).

Reaction systems studied were as follows:

1) System with exogenously added CDP-D-diglyceride contained: 50 µmoles of Tris-HCl buffer (pH 7.4), 5 µmoles of 2-mercaptoethanol, 2 mg of Triton-X100, 4 µmoles of $MgCl_2$, 0.15 µmole of L-glycero-3-phosphate-2-^{3}H (spec. activity 18.3 x 10^6 dpm/µmole), 0.5 µmole of CDP-D-diglyceride and 0.2 ml of mitochondria or microsomes (containing 2-4 mg of mitochondrial, or 5-6 mg of microsomal protein) in a final volume of 0.50 ml. After incubation at 37° for 60 minutes the reaction was stopped with 2.5 ml of methanol, and lipids were isolated according to Bligh-Dyer (9).

2) System endogenously generating CDP-D-diglyceride contained: 50 µmoles of K-phosphate buffer (pH 7.4), 1 µmole of CTP, 4 µmoles of ATP, 0.20 µmole of CoA, 1 µmole of $MgCl_2$, 1 µmole of $MnCl_2$, 0.1 µmole of Na-oleate, 0.5 µmole of L-glycero-3-phosphate-2-^{3}H (spec. activity 17.6 x 10^6 dpm/µmole) and 0.2 ml of mitochondria or microsomes (containing 4-6 mg of mitochondrial or 5-6 mg of microsomal protein) in a final volume of 0.50 ml. After incubation at 37° for

60 minutes the reaction was stopped with 2.5 ml of methanol, and the lipids were isolated according to Bligh-Dyer (9).

DEAE-cellulose column-, paper-, and thin-layer chromatography and determination of the radioactivity have already been described in detail along with other pertinent analytical methods (5).

RESULTS AND DISCUSSION

Results of this study obtained with system 1 and shown in Table I have established that liver mitochondria catalyze the conversion (dependent on exogenously added CDP-D-diglyceride) of L-glycero-3-phosphate-2-^{3}H to a major product, identified as phosphatidylglycerol, and to a minor product, phosphatidylglycerophosphate, according to the reaction scheme (I, a, b). Similar composition of lipids formed was obtained with liver microsomes, although the amount of synthesized labelled lipids was only 15% of the amount synthesized with mitochondria. With both mitochondria and microsomes, no detectable synthesis of cardiolipin was observed, as shown in Table I. These results are in agreement with results previously reported from this (4,5) and other laboratories (10,11) concerning the biosynthesis of phosphatidylglycerol in mammalian tissue.

Results shown in Table II, obtained with system 2, capable of generating CDP-D-diglyceride (6), have established the formation of cardiolipin. With this complete system in mitochondria (Table II, 1), phosphatidic acid was the major biosynthesized lipid, accompanied by phosphatidylglycerol, cardiolipin, phosphatidylcholine, and di- (and, possibly, mono-) glyceride. With purified mitochondria (Table II, 2) the major biosynthesized lipid was phosphatidylglycerol; however, the biosynthesis of cardiolipin was almost on the same level. The formation of cardiolipin was absolutely dependent on added CTP (Table II, 3). Without CTP the absence of the formation of phosphatidylglycerol was established (Table II, 3), strongly suggesting the involvement of this compound in the biosynthesis of cardiolipin, according to reaction scheme (I, c).

Table I

Formation and composition of labelled phospholipids from L-glycero-3-phosphate-2-^{3}H and exogenously added CDP-D-diglyceride in subcellular particles from guinea pig liver

Particle	Synthesis of labelled lipid (nmole/mg protein)	R_F	Composition (%) GPGP* 0.08	GP 0.13	GPGPG 0.26	GPG 0.42	G 0.62
1. Mitochondria	1.9		18.6	-	-	80.0	1.4
2. Mitochondria, purified	2.6		9.0	1.4	-	88.0	1.6
3. Mitochondria, purified, Triton-X100 omitted	1.4		25.7	-	-	71.6	2.7
4. Microsomes	0.3		3.6	2.7	-	92.2	1.5

Subcellular particles were incubated in the system supplied with exogenous CDP-D-diglyceride and L-glycero-3-phosphate-2-^{3}H (system 1 in the text); formed labelled lipids were isolated and analyzed as described in the text.

* Abbreviations: GPGP--glycerophosphorylglycerophosphate (phosphatidylglycerolphosphate), GP--glycerophosphate (phosphatidic acid), GPGPG--diglycerophorphorylglycerol (cardiolipin), GPG--glycerophosphorylglycerol (phosphatidylglycerol) and G--glycerol (di- and mono-glyceride).

Table II

Formation and composition of labelled lipids from L-glycero-3-phosphate-2-^{3}H in CDP-D-diglyceride generating system in subcellular particles from guinea pig liver

Particle	Synthesis of labelled lipids (nmole/mg protein)	Composition (%)						
		R_F GPGP* 0.08	GP 0.13	GPI 0.21	GPGPG 0.26	GPCh 0.32	GPG 0.42	G 0.62
1. Mitochondria, complete system	1.7	-	58.9	-	4.9	4.2	24.5	7.5
2. Mitochondria, purified, complete system	1.9	-	22.8	-	5.4	4.1	58.6	9.1
3. Mitochondria, purified, CTP omitted	1.5	-	36.3	-	-	52.2	-	11.5
4. Microsomes, complete system	23.0	-	27.8	7.5	-	21.3	2.7	40.7
5. Microsomes, CTP omitted	22.5	-	29.2	1.0	-	33.6	0.9	35.3

Subcellular particles were incubated in the system generating CDP-D-diglyceride and containing L-glycero-3-phosphate-2-^{3}H (system 2 in the text); formed labelled lipids were isolated and analyzed as described in the text.

* Abbreviations: Same as in Table I; GPI--glycerophosphorylinositol (phosphatidylinositol), and GPCh--glycerophosphorylcholine (phosphatidylcholine).

Table III

Composition of labelled lipids separated on DEAE-cellulose Column

Material	Composition (%)											
	Intact Lipids*						Water Soluble Products**					
	R_F	PhA 0.21	PhCh 0.48	PhG 0.64	Crdl 0.80	DiG 0.91	Unid.***	GP 0.13	GPGPG 0.26	GPCh 0.32	GPG 0.42	G 0.62	Unid.**
Peak A		-	38.0	-	-	56.9	5.1 (0.22)	-	-	40.5	-	59.5	-
Peak B		-	-	94.9	-	-	5.1 (0.12)	-	-	-	97.0	-	3.0 (0.18-0.20)
Peak C		95.1	-	-	4.9	-	-	92.2	7.8	-	-	-	-

Labelled lipids eluted from DEAE-cellulose column (Fig. 1, Peaks A, B, C) were subjected to t.l.c. (chloroform:methanol:conc. ammonium hydroxide - 65:25:4, v/v) in their intact form, and to paper chromatography (isopropanol:conc. ammonium hydroxide:water - 7:1:2, v/v) after mild alkaline hydrolysis, as described (5).

* Abbreviations: PhA--phosphatidic acid, PhCh--phosphatidylcholine, PhG--phosphatidylglycerol, Crdl--cardiolipin, DiG--di- (and mono-) glyceride.
** Abbreviations as in Tables I and II.
*** Unidentified, R_F value in parenthesis.

Microsomes, under identical conditions, were not able to catalyze the formation of a detectable amount of cardiolipin (Table II, 4, 5).

Further information on the nature of biosynthesized mitochondrial lipids was obtained as follows: Labelled lipids, obtained after the incubation of mitochondria in system 2 but on a scale 20x larger, were subjected to DEAE-cellulose column chromatography. Three distinct radioactive peaks (Fig. 1, A, B, C) were obtained; total recovery of radioactivity from the column was 94.8%. Labelled compounds were characterized by t.l.c. and paper chromatography. Results thus obtained (Table III), have shown that peak A contained phosphatidyl choline and di- (and mono-) glycerides. Peak B contained mainly phosphatidyl-glycerol, while peak C contained phosphatidic acid and cardiolipin. Although these latter lipids were not separated in their intact form on DEAE-cellulose column, mild alkaline hydrolysis of these lipids gave two distinctly separated compounds identified as (diglycerophosphoryl)glycerol and glycerophosphate. Additional evidence for the nature of (diglycerophosphoryl)glycerol was obtained by eluting this compound from the paper after separation from glycerophosphate and subjecting it to acetic acid hydrolysis (12). This hydrolysis gave a single radioactive, water soluble compound identified as glycerol-{-2-^{3}H}-1,3-diphosphate, a finding in agreement with the behaviour of (diglycerophosphoryl)-glycerol described previously (12).

Preparative thin layer chromatography of lipids (Fig. 1, C) (in solvent system chloroform:methanol:conc. ammonium hydroxide = 65:25:4, v/v) yielded pure cardiolipin (R_F = 0.80). After mild alkaline hydrolysis of cardiolipin thus obtained, only (diglycerophosphoryl)glycerol was detected.

Results described in this communication have established that mitochondria isolated from guinea pig liver are capable of catalyzing the formation of cardiolipin (diphosphatidylglycerol) utilizing endogenously formed CDP-D-diglyceride and, most likely, phosphatidylglycerol, according to the mechanism represented by scheme (I, c). This finding together with previously reported data demonstrating mitochondrial capability to synthesize phosphatidic and

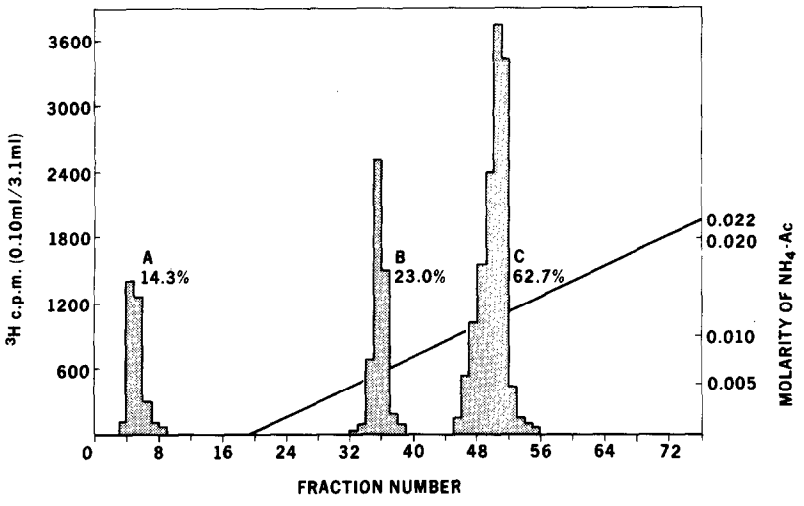

Fig. 1 DEAE-cellulose column chromatography of labelled lipids Mitochondria from guinea pig liver were incubated in a system described in the text. Labelled lipids isolated from this incubation were subjected to column chromatography as described (5). Numbers in the diagram indicate composition (%) based on the determination of radioactivity (^{3}H).

lyso-phosphatidic acid (13-17), CDP-D-diglyceride (18,19), phosphatidylglycerophosphate and phosphatidylglycerol (4,5,10,11), have established mitochondrial autonomy in the biosynthesis of all known polyglycerophosphatides.

In view of the finding of this study that the detected biosynthesis of cardiolipin takes place only in mitochondria generating CDP-D-diglyceride, the following implications seem justified: 1) Cardiolipin synthetase has high specificity for the endogenously formed CDP-D-diglyceride, and/or 2) Biosynthesis of polyglycerophosphatides in mitochondria is highly compartmentalized. The biosynthesis of cardiolipin is associated with a compartment capable of generating CDP-D-diglyceride, or permeable to the endogenously generated CDP-D-diglyceride, but inaccesible to the exogenously added CDP-D-diglyceride, while the biosynthesis of phosphatidylglycerol is associated with a compartment permeable to the exogenously supplied CDP-D-diglyceride. The compartment involved in the biosynthesis of cardiolipin is apparently permeable to the synthesized phosphatidylglycerol; it could also be an additional site for the synthesis of phosphatidylglycerol in mitochondria. Experiments currently in

progress in this laboratory are designed to provide more information on these possibilities, as well as on the relation between the biosynthesis of polyglycerophosphatides and mitochondrial inner and outer membranes.

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