# THE MECHANISM OF THE BIOSYNTHESIS OF CARDIOLIPIN IN MITOCHONDRIA

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## SUMMARY

The mechanism of the biosynthesis of cardiolipin in isolated rat liver mitochondria was studied using phosphatidylglycerol-2'-$^{3}H$ and CDP-diglyceride-2-$^{14}C$. Cardiolipin enzymatically synthesized contained both isotopes and was formed without an apparent release of glycerol-2-$^{3}H$. These results have established that the reaction mechanism involving both phosphatidylglycerol and CDP-diglyceride in the biosynthesis of cardiolipin is operational in mitochondria.

## INTRODUCTION

Two mechanisms for the biosynthesis of cardiolipin can be represented by Reaction schemes 1 and 2.

Phosphatidylglycerol + CDP-diglyceride = Cardiolipin + CMP.....(1)

2 Phosphatidylglycerol = Cardiolipin + Glycerol.....(2)

The formation of cardiolipin from phosphatidylglycerol according to Reaction scheme 1, originally described by Stanacev, Chang and Kennedy (1), should be absolutely dependent on CDP-diglyceride, since CDP-diglyceride is a phosphatidyl-donor while phosphatidylglycerol is a phosphatidyl-acceptor of the phosphatidyl-group. The formation of cardiolipin from phosphatidylglycerol according to Reaction scheme 2, originally described by Stanacev and Stuhne-Sekalec (2), is a process of intermolecular transphosphatidylation and should not require CDP-diglyceride; phosphatidylglycerol is the donor and the acceptor of the phosphatidyl-group.

A survey of rapidly growing literature on the biochemistry of cardiolipin

shows that phosphatidylglycerol is generally recognized as the precursor of cardiolipin biosynthesis on the basis of convincing experimental evidence. No direct evidence has been published, however, which would demonstrate the incorporation of CDP-diglyceride into cardiolipin in a reaction starting from phosphatidylglycerol.

Stanacev et al (1) have shown the stimulation of cardiolipin biosynthesis by CDP-diglyceride in Escherichia coli. Davidson and Stanacev (3) have demonstrated mitochondrial ability to synthesize cardiolipin in a system starting from sn-glycero-3-phosphate; they have established the dependence of this synthesis on CTP and suggested CDP-diglyceride involvement. Hostetler et al (4) have also shown the stimulation of cardiolipin biosynthesis by CDP-diglyceride. These data indirectly support Reaction mechanism 1 for the biosynthesis of cardiolipin.

Studying various aspects of cardiolipin biochemistry in different bacteria, De Siervo and Salton (5), Polonovski et al (6), and Short and White (7) have reported experiments compatible with Reaction mechanism 2 for the biosynthesis of cardiolipin.

Most recently, Hirschberg and Kennedy (8) have described the experimental evidence for biosynthesis of cardiolipin in Escherichia coli according to Reaction scheme 2, attributing to the CDP-diglyceride a stimulative role only, since no phosphatidyl transfer from added CDP-diglyceride to phosphatidylglycerol could be demonstrated, although cardiolipin was formed.

We wish to present now direct experimental evidence in support of Reaction mechanism 1 for the biosynthesis of cardiolipin in isolated rat liver mitochondria. The evidence is based on the incorporation of both phosphatidylglycerol-2'-$^{3}$H and CDP-diglyceride-2-$^{14}$C into cardiolipin without the liberation of glycerol-2-$^{3}$H, establishing the participation of both substrates in the enzymatic formation of cardiolipin.

## MATERIALS AND METHODS

Rat liver mitochondria were prepared essentially as described (9) using

a medium containing 0.25 M sucrose, 0.001 M EDTA, 0.010 M 2-mercaptoethanol and 0.010 M Tris-HCl buffer (pH 7.2). The microsomal content was determined by the glucose-6-phosphatase assay (10) and by the NADPH-cytochrome c reductase assay (11) and was found to be 5-7% and 3-5%, respectively.

Synthetic CDP-diglyceride (containing 77% oleic acid) was purchased from Serdary Research Labs., London, Ontario and was converted into Tris-salt before use. CDP-diglyceride-2-$^{14}$C was prepared from phosphatidic acid-2-$^{14}$C and CMP-morpholidate according to the method of Agranoff and Suomi (12) and was purified by DEAE-cellulose column chromatography essentially using the described technique (13). It was checked for purity by TLC and for enzymatic activity by CDP-diglyceride dependent uptake of sn-glycero-3-phosphate according to the described assay (14). Phosphatidic acid-2-$^{14}$C was obtained enzymatically from sn-glycero-3-phosphate-2-$^{14}$C and palmitate according to the described procedure (15).

The incubation was carried out in two stages. The incubation mixture in the Ist stage, similar to that already described (16), contained: 50 µmoles of Tris-HCl buffer (pH 7.4), 5 µmoles of 2-mercaptoethanol, 1.0 µmole of sn-glycero-3-phosphate-2-$^{3}$H (spec. activity 1.92 x $10^6$ cpm/µmole), 100 µmoles of CDP-diglyceride (Serdary Research Labs., London, Ont.) and 0.12 ml of rat liver mitochondria (containing 6.0 mg of protein) in a final volume of 0.50 ml. After incubation for 2 hours at 37°, the reaction mixture was placed in ice, layered on the top of 30.0 ml ice-cold 0.30 M sucrose and centrifuged at 70,000 x g for 30 minutes, essentially as described for the isolation of membrane-bound phosphatides (17). Pellets thus obtained from two incubation mixtures were combined by resuspension in 0.10 ml of Tris-HCl buffer (pH 7.4) and used for the IInd incubation. The incubation mixture in the IInd stage contained: 50 µmoles of CDP-diglyceride-2-$^{14}$C (spec. activity 2.98 x $10^6$ cpm/µmole), two combined pellets from the Ist incubation resuspended in 0.10 ml Tris-HCl (pH 7.4) and 80 µmoles of $MnCl_2$ (added last) in a final volume of 0.50 ml. After incubation for 1 hour at 37°, the reaction was stopped with 2.5 ml of methanol

and lipids were isolated according to the method of Bligh-Dyer (18). An aliquot of both chloroform and water phase was assayed for radioactivity as described (13). Labelled lipids were subjected to mild alkaline hydrolysis and water-soluble compounds were separated and characterized by paper chromatography using procedures already described (3,13).

## RESULTS

The experimental approach and the results obtained are shown in Table I. In order to avoid the problem of the utilization of a water-insoluble substrate such as phosphatidylglycerol in an enzymatic reaction, this substrate was synthesized *in situ* from sn-glycero-3-phosphate-2-$^{3}H$ and CDP-diglyceride in mitochondria; however, it was not extracted from the mitochondria, but was isolated in the form of a membrane-bound lipid by passing the mitochondria, after incubation, through sucrose. The phosphatidylglycerol-2'-$^{3}H$ obtained in this manner was analyzed before further incubation, and results shown in Table I, 1, have established that it was 97.68% radioactively pure. Furthermore, the amount of cardiolipin-$^{3}H$ formed in the Ist incubation stage was almost negligible (0.29%) as shown in Table I, 1. When the mitochondrial preparation thus obtained, carrying the membrane-bound phosphatidylglycerol-2'-$^{3}H$, was re-incubated in the IInd stage with CDP-diglyceride-2-$^{14}C$ in the presence of $MnCl_2$, newly synthesized cardiolipin amounted to 17.85% of $^{3}H$-labelled lipids; it contained both $^{3}H$ and $^{14}C$ isotopes in a ratio of 0.67, as shown in Table I, 2, therefore directly establishing the participation of both phosphatidylglycerol-2'-$^{3}H$ and CDP-diglyceride-2-$^{14}C$ in this synthesis. The amount of water-soluble $^{3}H$-compound(s) in the water-phase after Bligh-Dyer isolation of lipids (18) was established as well. It was found that while 2.62 µmoles of cardiolipin based on $^{3}H$, was formed (Table I, 2), 1.43 µmoles of $^{3}H$-compound(s) was released into water-soluble form, out of which only 7-10% was in the form of glycerol-2-$^{3}H$, as was further established by paper chromatography (13). The

Table I

Incorporation of phosphatidylglycerol-2'-$^{3}H$ and CDP-diglyceride-2-$^{14}C$ into cardiolipin

| Incubation system | Total lipids ($^{3}H$) nmoles | Phosphatidylglycerol-2'-$^{3}H$* | | Cardiolipin* | | |
|---|---|---|---|---|---|---|
| | | nmoles | % of total lipids ($^{3}H$) | nmoles $^{3}H$ | nmoles $^{14}C$ | % of Total lipids ($^{3}H$) |
| 1. Ist incubation only, complete system | 17.99 | 17.57 | 97.68 | 0.05 | - | 0.29 |
| 2. Ist incubation, complete system; IInd incubation, complete system | 14.72 | 11.21 | 76.20 | 2.62 | 1.76 | 17.85 |
| 3. Ist incubation, complete system; IInd incubation, CDP-diglyceride-$^{14}C$ replaced with 100 nmoles of CDP-diglyceride | 17.41 | 13.91 | 79.93 | 3.12 | - | 17.96 |
| 4. Ist incubation, complete system; IInd incubation, omit both CDP-diglyceride-$^{14}C$ and $MnCl_2$ | 18.13 | 17.95 | 98.67 | 0.07 | - | 0.40 |
| 5. Ist incubation, complete system; IInd incubation, omit $MnCl_2$ | 17.50 | 16.67 | 95.28 | 0.02 | - | 0.12 |

The incubation was carried out in two stages; experimental details are described under Materials and Methods.

* Determined as glycerophosphoryl-glycerol(-2'-$^{3}H$) and glycerophosphoryl-glycero(-2'-$^{3}H$)phosphoryl-glycerol(-2"-$^{14}C$) respectively, obtained after mild alkaline hydrolysis of lipids (3) and separated by paper chromatography in iso-propanol:conc. ammonia:water = 28:4:8 (v/v/v) as described (3,13).

amount of water-soluble $^{3}H$-compound(s) was too low to satisfy the stoichiometry of Reaction mechanism 2.

Results shown in Table I, 2, obtained with CDP-diglyceride instead of CDP-diglyceride-2-$^{14}C$, confirmed the formation of cardiolipin in the same amount (17.96% as compared to 17.85%). The dependence of the biosynthesis of cardiolipin on CDP-diglyceride and $MnCl_2$ is shown in Table I, 4. When these compounds were omitted, the negligible amount of cardiolipin was detected. In addition, a requirement of this synthesis on added $Mn^{++}$ was also established, as shown in Table I, 5. Results not shown here have established that $Mg^{++}$ in approximately the same concentration can replace $Mn^{++}$ with the same effect.

Additional evidence for the incorporation of CDP-diglyceride-2-$^{14}C$ into cardiolipin was obtained from the experiment where sn-glycero-3-phosphate-2-$^{3}H$ was replaced with 2.0 μmoles of rac-glycero-3-phosphate in an incubation mixture otherwise identical to that described under Materials and Methods. After following the identical procedure to that described for the experiments listed in Table I, $^{14}C$-lipids were separated on TLC (in chloroform:methanol: conc. ammonia = 65:25:4, v/v/v) and cardiolipin-$^{14}C$ ($R_f$ = 0.53, 11.4% of applied $^{14}C$-compounds) was eluted and subjected to mild alkaline hydrolysis (3). Paper chromatography of compounds thus obtained established that 91.6% of water soluble $^{14}C$-compounds was in the form of (diglycerophosphoryl)glycerol i.e. deacylated cardiolipin.

## DISCUSSION

Experiments described in this communication have demonstrated, for the first time, the direct participation of both phosphatidylglycerol and CDP-diglyceride in the enzymatic formation of cardiolipin according to Reaction mechanism 1. Under described experimental conditions, the presence of CDP-diglyceride:phosphatidylglycerol phosphatidyl transferase was demonstrated in isolated mitochondria. These results and results already reported (2-8) have established that the biosynthesis of cardiolipin can take place according to both mechanisms represented by Reaction schemes 1 and 2. Whether mechanism

1 is the only pathway for the biosynthesis of cardiolipin in mammalian tissue, and mechanism 2 the only pathway for this biosynthesis in bacteria, or whether both mechanisms are operational in mammalian tissue and in bacteria under appropriate conditions, remains to be established. These aspects of cardiolipin biochemistry with their implications are currently under experimental investigation in this laboratory and will be reported elsewhere.

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