CARDIOLIPIN FROM BEEF HEART MITOCHONDRIA: FATTY ACID POSITIONING AND MOLECULAR SPECIES DISTRIBUTION

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SUMMARY

The positioning of fatty acids within cardiolipin from beef heart mitochondria was determined and compared to phosphatidyl choline and phosphatidyl ethanolamine from the same source. Unsaturated fatty acids were preferentially esterified in the β-position and saturated fatty acids were contained preferentially in the α-position of the choline and ethanolamine phosphatides. In contrast, fatty acids were nearly randomly distributed between the α- and β-positions of cardiolipin. On acetolysis, cardiolipin yielded diglyceride acetates which were separated into three distinct species, trienes, tetraenes and pentaenes, by argentation thin-layer chromatography. Tetraenes accounted for more than 75% of the total diglyceride acetates and contained 94% linoleic acid, suggesting the existence of a molecular species of cardiolipin containing solely linoleic acid.

In mammalian systems, cardiolipin (diphosphatidyl glycerol) occurs virtually exclusively in mitochondria (1). Although the fatty acid composition of cardiolipin has been determined repeatedly, there is still controversy over the positional distribution of fatty acids within the cardiolipin molecule. Marinetti (2), by phospholipase A hydrolysis of a commercial cardiolipin preparation, found an essentially random distribution of fatty acids between the α- and β-positions. Others have observed a degree of selectivity of fatty acids for either the α- or β-positions of the molecule (3-6). With cardiolipin from brain grey matter (5), bovine and human heart (3) and rat liver (6), palmitic and linoleic acids show some preference for the α-position and oleic acid is preferentially contained in the β-position. As yet there have been no attempts to separate and determine the acyl groupings present in different molecular species of cardiolipin.

It has recently been demonstrated that a small amount of cardiolipin is tightly bound to cytochrome oxidase from beef heart mitochondria (7). This lipid, which is essential for activity of the enzyme, has a fatty acid composition very similar to that of the total mitochondrial cardiolipin. These observations indicated the need for a study of the fatty acid positioning and molecular species distribution of cardiolipin as a first step toward eventual determination of the reason for the preferential binding of cardiolipin to cytochrome oxidase.

METHODS

Beef heart mitochondria were prepared by the method of Low and Vallin (8). Lipids were recovered by extraction with chloroform:methanol (2:1, v/v) followed by chloroform:methanol:aqueous ammonia (7:1:5%). The combined extracts were washed (9), concentrated *in vacuo* without heating and stored in a nitrogen atmosphere at -20° until analysis. A small amount of butylated hydroxyanisole (BHA) was added to prevent lipid oxidation.

Cardiolipin, phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) were isolated by preparative thin-layer chromatography (TLC) on 1 mm thick silica gel G plates developed in a mixture of chloroform:methanol:water (70:22:3, by vol.) and the lipid bands were localized by spraying the edges of the plate with dichlorofluorescein. Lipids were recovered from unstained portions of chromatoplates by elution of the silica gel scrapings with chloroform:methanol:water (12:8:1) (10). Analysis of the recovered fractions by two-dimensional TLC (11) revealed that PE and cardiolipin were free of other lipid contaminants and that the PC fraction contained approximately 5% phosphatidyl inositol as the only contaminant.

PC and PE were digested with phospholipase A (phosphatide acyl-hydrolase, E.C. 3.1.1.4) essentially according to Kuksis *et al.* (12). Twenty mg of the lipid, dissolved in 15 ml of diethyl ether, was added to 15 ml of the buffered enzyme solution containing 2 mg of heat treated (13) *Naja naja* venom protein.

This mixture was purged with nitrogen, sealed and incubated for 2 to 4 hr at 30° with shaking. Reactions were stopped by extraction with chloroform:methanol (2:1). TLC analysis of extracts showed that hydrolysis was complete under these conditions. For digestion of cardiolipin, the above reaction mixture contained, in addition, 2 mg of sodium taurocholate and 1 mg BHA and incubation was continued for 18 to 24 hr. Alternatively, 20 mg each of cardiolipin and PC were dissolved in 50 ml diethyl ether containing 0.2 ml enzyme solution (2 mg protein) and incubated for 16 hr at 30° under pure nitrogen in a Dubnoff metabolic incubator (2). Lysolipids and free fatty acids were recovered separately by TLC in chloroform:methanol:acetic acid;water (65:25:8:4) and petroleum ether:diethyl ether:acetic acid (85:15:1) respectively.

Cardiolipin was converted into diglyceride acetates by acetolysis according to Renkonen (14). Approximately 150 mg of cardiolipin, 2 ml of acetic anhydride-acetic acid (2:3) and 2 mg of BHA were sealed under nitrogen in an ampoule and heated at 145° for 40 hr. The reaction mixture was cooled, evaporated to dryness and partitioned in chloroform:methanol:water (8:4:3). TLC analysis showed the reaction to be complete. Diglyceride acetates were purified by preparative TLC in the solvent system described for recovery of free fatty acids. The diglyceride acetates were subfractionated according to total number of double bonds by chromatography on plates coated with silica gel G-silver nitrate (10:3, by weight). Plates were developed in chloroform: methanol (99:1) and lipid bands were located by spraying with dichlorofluorescein.

Methyl esters were prepared by treatment with boron trifluoride-methanol (15) and analyzed by gas chromatography as described previously (11). All solvents were redistilled in glass prior to use. TLC solvent systems contained small amounts of BHA and plates were dried in a nitrogen atmosphere to minimize lipid oxidation.

RESULTS AND DISCUSSION

With natural PC and PE, it is the general finding that the β-linked fatty acids are predominantly unsaturated and that the α-linked acids are

TABLE I

Composition and Positional Distribution of Fatty Acids of Beef Heart Mitochondrial Phospholipids (% by weight)

	PC			PE			Cardiolipin			
Acid	Total	LPC	FFA	Total	LPE	FFA	Total[a]	Total[b]	Lyso-1[c]	Lyso-2[c]
14:0	7.3	7.5	4.2	3.3	7.9	0.8	0.4	0.4	0.5	2.0
14:1	0.1	--	2.2	--	--	--	0.1	0.2	0.3	1.3
16:0	21.9	75.0	13.5	6.8	26.7	2.8	1.6	2.9	3.3	8.1
16:1	1.2	--	4.5	--	--	--	3.2	2.8	2.8	5.4
18:0	6.0	14.5	2.6	29.3	60.6	1.1	1.2	2.4	1.7	5.0
18:1	15.2	0.6	18.5	2.8	--	2.8	7.6	11.4	6.8	3.9
18:2	30.6	0.7	33.7	11.6	3.6	15.6	79.8	74.9	79.7	68.9
18:3	2.9	0.9	3.5	1.3	1.2	1.5	4.0	5.0	4.8	5.4
20:3	5.3	--	4.5	4.6	--	4.2	1.2	--	--	--
20:4	7.8	0.7	8.5	34.4	--	59.3	1.0	--	--	--
20:5	1.1	--	2.9	4.2	--	8.5	--	--	--	--
22:5	0.6	--	1.4	1.8	--	3.3	--	--	--	--

Abbreviations: PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; LPC, lysophosphatidyl choline; LPE, lysophosphatidyl ethanolamine; FFA, free fatty acids. Fatty acids are abbreviated as number of carbons:number of double bonds.

[a]Composition of cardiolipin before incubation.

[b]Composition of cardiolipin after incubation without enzyme.

[c]Lyso-1 and Lyso-2 refer to lysocardiolipins with one and two fatty acids removed, respectively.

predominantly saturated. Such was found to be the case with the choline and ethanolamine phosphatides from beef heart mitochondria (Table I) where there was a clear preference for the more unsaturated fatty acids to occupy the β-position. Saturated acids were contained preferentially in the α-position.

With the less unsaturated PC, fatty acids with two or more double bonds were nearly completely absent from the β-position. PE, in which 61% of the fatty acyl residues were unsaturated, contained relatively low levels of unsaturated fatty acids in the α-position.

Such a distinctly nonrandom distribution of fatty acids clearly cannot be the case with beef heart cardiolipin inasmuch as this phosphatide contains 80% linoleic acid and only 3.2% saturated acids (Table I). Cardiolipin from liver and kidney also contain linoleic acid levels of this magnitude (4, 16). Phospholipase A acts upon cardiolipin, liberating only the β-linked fatty acids from both diglyceride parts (17). Although the reaction proceeds slowly, addition of PC greatly accelerates hydrolysis (2, 17). In the present study it was observed that cardiolipin was completely degraded to the lyso derivative containing two fatty acids in 16 hr at 30° if PC was present in the reaction mixture. However, there was some loss of unsaturated fatty acids under these conditions, even though an antioxidant was present and the reaction was carried out in a nitrogen atmosphere (Table I). There was also a small amount of chemical hydrolysis of cardiolipin when incubated in moist ether without enzyme. By interrupting the enzymatic reaction at earlier time periods a lyso derivative containing 3 fatty acids could be isolated. The fatty acid composition of these lysocardiolipins is given in Table I. Fatty acid patterns of these hydrolysis products were similar to each other and to the cardiolipin incubated without enzyme. There appeared to be a slight preference for the 16:0 and 16:1 acids in the α-position and for the 18:1 acid in the β-position. Since some of the differences noted can be ascribed to degradation of unsaturated acids, the results indicate a nearly random positioning of fatty acids within the cardiolipin molecule. Several attempts were made to hydrolyze cardiolipin in the absence of other lipids. In all cases hydrolysis proceeded slowly and was never more than 30% complete in 24 hr at either 30 or 37°. Nevertheless, in all cases analysis of the lyso derivatives and free fatty acids revealed a slight preference of the 16:0 and 16:1 acids for the α-position

TABLE II

Fatty Acid Composition of Diglyceride Acetates from Beef Heart Mitochondrial Cardiolipin (% by weight)

		Diglyceride Acetates			
Acid	Cardiolipin	Total	Trienes	Tetraenes	Pentaenes
14:0	0.2	0.2	0.8	0.5	1.9
16:0	0.7	0.9	3.2	1.7	6.8
16:1	2.3	2.6	11.1	0.6	5.4
18:0	0.5	0.4	1.3	1.0	4.6
18:1	5.5	6.6	30.3	0.9	6.6
18:2	79.0	80.5	52.1	93.6	39.3
18:3	5.6	5.3	1.2	0.8	23.8
20:3	5.0	2.6	--	0.4	6.6
20:4	1.2	0.9	--	0.6	5.1

Fatty acids abbreviated as number of carbons:number of double bonds. Trienes, tetraenes and pentaenes refer to the total number of double bonds in the fatty acid moietes of the molecule.

and of the 18:1 acid for the β-position. In no case was any selectivity of 18:2 for either position evident.

The high levels of linoleic acid and its apparently random distribution indicated the possible occurrence of a species of cardiolipin containing solely linoleic acid. Diglyceride acetates were prepared from cardiolipin by acetolysis. Under the conditions employed, cardiolipin was totally degraded to diglyceride acetates. Although there was some oxidative degradation, as evidenced by darkening of the reaction mixture, colorless diglyceride acetates could be isolated by TLC which had a fatty acid composition virtually identical to that of the original cardiolipin (Table II). Renkonen (14) has demonstrated that intermolecular migration and cis-trans isomerization do not occur under

these conditions. Argentation TLC revealed the presence of only three molecular species of diglyceride acetates. By their TLC behaviour and gas chromatographic analysis of their component fatty acids (Table II), these were identified as trienes, tetraenes and pentaenes. The tetraene fraction accounted for more than 75% of the diglyceride acetates. Linoleic acid accounted for 94% of the acyl groupings in the tetraene fraction (Table II). This indicates the predominance of diglyceride acetates containing solely linoleic acid and, since tetraenes account for such a large proportion of the total, strongly implies the existence of a cardiolipin species containing entirely linoleic acid. To our knowledge, existence of monoacid phosphoglycerides has not been observed in mammalian systems. Linoleate was also the major acid in the triene and pentaene fractions. Trienes also contained large amounts of palmitoleic and oleic acids and the pentaene fraction contained most of the 18:3, 20:3 and 20:4 acids of the diglyceride acetates. The predominance of a tetraene fraction composed largely of linoleic acid is in agreement with the report of Wood and Harlow (6) who, by gas chromatographic analysis, found that a diglyceride acetate containing two 18 carbon chain length fatty acids represented 70% of the diglycerides derived from cardiolipin of rat liver.

Results reported herein suggest the existence of a molecular species of cardiolipin containing solely linoleic acid. The data also suggest another molecular species in which one of the diglycerides contains two linoleic acid residues and the other diglyceride contains one linoleic acid residue along with some other acyl grouping. This raises some interesting possibilities for binding of cardiolipin to protein through induced dipoles at the double bonds and may partially explain the tenaceous binding of cardiolipin to cytochrome oxidase (7). Since it has been found that approximately 50% of the cardiolipin of beef heart is complexed with protein (18), proteolipids containing cardiolipin may be of great importance in determining structure and function of mitochondrial membranes. Finally, these results show that the origin of the tightly bound saturated lipids in cytochrome oxidase which we have reported is

unlikely to be from the bulk of the cardiolipin. The contrast between the extensive unsaturation in cardiolipin and the saturation in the unknown compounds is noteworthy (7).

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