Phospholipids of Adrenal Cortex Mitochondria and the Steroid Hydroxylases: The Lipid-Environment of Cytochrome P-450

by

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Summary: Adrenal cortex mitochondria have a high content of phospholipids with elevated ratios of phosphatidyl ethanolamine and phosphatidyl choline to diphosphatidyl glycerol, as compared with those of heart, liver, and kidney mitochondria. An extracted cytochrome P-450 preparation contained phosphatidyl ethanolamine and phosphatidyl choline as major components but no diphosphatidyl glycerol. After phospholipase-C pretreatment of the P-450 preparation, the reconstituted steroid 11 β-hydroxylase activity was inhibited, whereas the cholesterol desmolase activity was activated to a comparable extent.

Phospholipids are essential components of all mitochondria. Although the mitochondrial phospholipid content varies from organ to organ, the relative ratios of the major phospholipids are fairly constant in mammalian tissues, PE : PC : PI = 1 : 1 : 0.1 (1). In highly specialized tissues such as insect flight muscle, however, the ratios of various phospholipids can be vastly different, PE : PC : PI = 4.6 : 1 : 1.2 (2). In the case of adrenal cortex, the mitochondria are different from the mitochondria of other tissues in that they have unique vesicular ultrastructure and contain steroid hydroxylases with P-450 as the terminal oxidase. Available evidence for the requirement of phospholipids by steroid hydroxylases is somewhat contradictory. Ungar and his colleagues (3) reported that PC inhibits the activity of adrenal cholesterol desmolase, and Mason and Boyd (4) described the inhibitory effect of PC in human term placental cholesterol desmolase activity. On the other hand, Péron (5) suggested that the phospholipid-cholesterol complex serves as a normal substrate for adrenal steroidogenesis. The purpose of this investigation is to clarify which, if any, phospholipid is necessary for adrenal mitochondrial steroid

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This study was supported by Research Grants from the National Institutes of Health (AM-12713, AM-13724, and AM-5384). The abbreviations used are as follows : PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PS, phosphatidyl serine; PI, phosphatidyl inositol; DG, diphosphatidyl glycerol, and P-450, cytochrome P-450.

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hydroxylases and to gain some insight into the state of the lipid-environment of P-450 molecules.

Methods and Materials

Highly purified bovine adrenal cortex mitochondria were prepared as described previously (6). Contamination of microsomal enzymes into the mitochondrial fraction was less than 7%. Extraction and quantitative determination of various phospholipids after two-dimensional thin layer chromatography were as described by Fleischer et al. (1). Cytochromes were determined with an Aminco-Chance dual wavelength spectrophotometer after reduction by 5 mM succinate in the presence of 1 mM HCN. P-450 was determined according to Cammer and Estabrook (7). Iron-sulfur protein was determined by its reduced EPR signal intensity at $g = 1.94$ using purified adrenal iron-sulfur protein (adrenodoxin) as a standard. EPR spectroscopy was carried out by the use of a Varian E-4 spectrometer. The assays for steroid hydroxylases were described previously (8, 9). Phospholipase-C from Bacillus cereus was a kind gift from Prof. Roy Vagelos. P-450 particles were extracted from sonicated mitochondria with Na-cholate (0.5 mg/mg mitochondrial protein) at 4° with stirring for 60 minutes. After centrifugation at 105,000 x g for 60 minutes, the clear supernatant which contained P-450 was collected and Na-cholate was removed by passing through a sephadex G-25 column. Adrenal iron-sulfur protein and its reductase were prepared as described previously (8, 10).

Results

Phospholipids content in bovine adrenal cortex mitochondria : The phospholipid composition of the adrenal cortex mitochondria is compared to that of heart, liver and kidney mitochondria in Table I. The salient features are briefly summarized below. (i) The adrenal cortex mitochondria have a higher phospholipid content per mg of mitochondrial protein than other mitochondria, and (ii) the adrenal mitochondrial phospholipids have a lower percentage of DG, higher percentage of PI, and higher PE/DG, and PC/DG, compared with those of other mitochondria.

Phospholipid composition of extracted cytochrome P-450 particles : A thin-layer chromatogram of phospholipids from extracted P-450 particles with and without treatment of phospholipase-C is shown in Fig. 1. It is clear that P-450 particles have PE and PC as major components but no DG. Upon treatment with phospholipase-C, there is a drastic reduction of PE and PC. The content of phospholipids in P-450 particles ranged from 350 to 450 nmoles of total phospholipid P per mg protein or from 175 to 225 nmoles per nmole of P-450. The ratio of PE to PC was about unity.

Table I Composition of Phospholipids in Adrenal Cortex Mitochondria

Mitochondria	Total P nmoles / mg protein	Phospholipids DG %	PE %	PC %	PI %	PE/DG mole/mole	PC/DG mole/mole	aa_3 nmoles / mg protein	P-450 nmoles / mg protein	Fe-S ng-atoms / mg protein
Adrenal	445—454	12.9—14.1	37.5—41.7	39.8—43.8	3.0—5.0	5.88	6.14	0.24—0.28	1.5—2.2	2.04
Heart	371	20.2—20.6	35.5—35.7	40.1—41.1	1.1—1.5	3.50	2.98	---	absent	absent****
Kidney*	245	19.2±0.4	38.1±0.4	39.6±0.3	---	2.90	4.28	0.23	?**	absent
Liver*	187	17.2±0.5	34.5±0.4	43.4±0.3	---	4.04	5.02	0.22***	absent	absent
Heart*	---	20.6±0.4	35.5±0.4	40.3±0.5	---	3.30	3.64	1.3	absent	absent

*Fleischer et al. (1)
**Gray et al. (12)
***Klingenberg (13)
****refer to ferredoxin-like protein

Adrenal and heart mitochondria, approximately 30 mg protein, were extracted with chloroform : methanol (2 : 1, v/v) under a nitrogen atmosphere and in the presence of an antioxidant (BHT). After removal of nonlipid, aliquots of the concentrated extract were taken for the determination of total phospholipid phosphorus and for phospholipid composition by two-dimensional thin-layer chromatography. The solvent systems were chloroform : methanol : 28% aqueous ammonia (65 : 35 : 5) and chloroform : acetone : methanol : acetic acid : water (5 : 2 : 1 : 1 : 0.5). The spots were detected by charring.

Heart mitochondria were prepared by the method of Crane et al. (15).

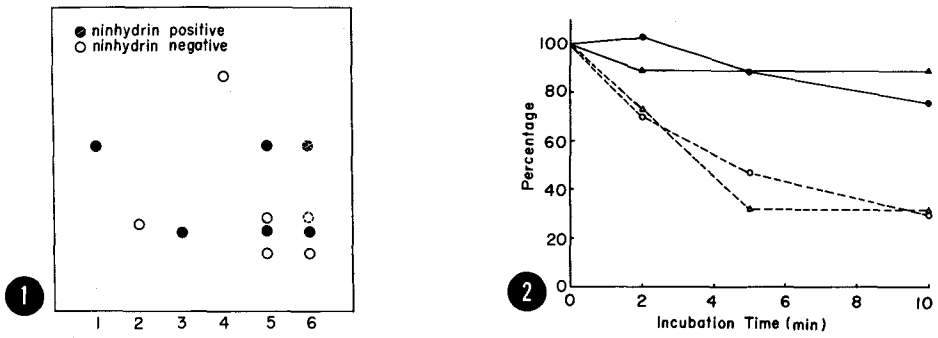

Fig. 1 Thin Layer Chromatogram of Phospholipids from Extracted P-450 Particles with and without Phospholipase-C Treatment

1, 2, 3, and 4 : standard PE, PC, PS, and DG, respectively; 5 and 6 : phospholipids from the extracted P-450 sample with and without phospholipase-C treatment, respectively. 3.4 nmoles of the P-450 was incubated in a reaction mixture of 2.1 ml which contains 0.1 unit of phospholipase-C when added, 20 μmoles of phosphate buffer (pH 7.4), and 2 μmoles of dithiothreitol at 37° for 5 minutes. The chromatogram was developed by a solvent consisting of chloroform : methanol : acetone : acetic acid : water (5 : 2 : 1 : 1 : 0.5) (14). Other manipulations were similar to those described in Table I.

Dotted circles represent faint spots. All spots were detected by charring, and the shadowed spots were ninhydrin-positive.

Fig. 2 Disappearance of Phospholipids and Steroid 11 β-hydroxylase Activity by Phospholipase-C Treatment

○ : Steroid 11 β-hydroxylase activity with the extracted P-450 particles pretreated by phospholipase-C
● : the same without the pretreatment
△ : phospholipid phosphorus remaining after pretreatment of the P-450 by phospholipase-C
▲ : the same without the pretreatment

2.4 nmoles of the P-450 was incubated in a reaction mixture of 3.1 ml, which contained 0.2 units of phospholipase-C, 30 μmoles of phosphate buffer (pH 7.4), and 1 μmole of dithiothreitol at 37° for the period indicated. The reaction was terminated by adding 10 μmoles of EDTA. For the determination of steroid 11β-hydroxylase activities, the reaction mixture (2.4 ml) contained 1.70 nmoles of P-450, 3.3 nmoles of adrenal iron-sulfur protein, 0.33 nmoles of its reductase, 0.58 μmoles of NADP, 10 μmoles of glucose-6-phosphate, 5 units of glucose-6-phosphate dehydrogenase, 0.2 μmoles of deoxycorticosterone, and 24 μmoles of phosphate buffer (pH 7.4). The reaction was carried out at 37° for 60 minutes. For the determination of phospholipid phosphorus, phospholipids from the treated and untreated P-450 samples were extracted by the method of Blogh and Dyer (16) and subjected to the analysis of phosphorus (17).

Effects of phospholipase-C on adrenal cortex mitochondrial steroid hydroxylases : Effects of phospholipase-C pretreatment of an extracted P-450 particles

Table II Effect of Phospholipase-C Treatment on Steroid Hydroxylase Activities

Experiment	Phospholipase-C Added	11 β-Hydroxylase		Cholesterol Desmolase		Phosphorus in Lipids	
	units	nmoles / 60 min	%	nmoles / 60 min	%	nmoles	%
I	0	19	100	2.8	100	760	100
	0.1	14.2	75	3.9	139	450	59
II	0	13.5	100	3.1	100	800	100
	0.1	9.8	73	4.4	140	500	63

3.0 and 3.4 nmoles of P-450 were pretreated with 0.1 unit of phospholipase-C at 37° for 5 minutes in Experiment I and II, respectively. Reactions were terminated by adding 10 μmoles of EDTA.

The enzyme assays and the phospholipid phosphorus determination were described in Fig. 2. For cholesterol desmolase activity measurements, 8.5 nmoles of 26-^{14}C-cholesterol was used instead of deoxycorticosterone.

on the reconstituted steroid 11 β-hydroxylase activity are shown in Fig. 2. There was a parallel decrease in the 11 β-hydroxylase activity and the phospholipid content, suggesting that decline of the enzymatic activity is due to the removal of phospholipids. The products of the phospholipase-C reaction with the mitochondrial lipids had no effect on the hydroxylase activity. Boiled phospholipase-C sample had no effect on either the enzymatic activity or phospholipid content. In contrast to the 11 β-hydroxylase activity, reconstituted cholesterol desmolase activity was not inhibited by the phospholipase-C pretreatment of P-450. Indeed, under conditions where there was approximately a 40% decrease in phospholipid content, and a 25% decrease in 11 β-hydroxylase activity, there was also approximately a 40% increase in the cholesterol desmolase activity (Table II).

Discussion

Our investigation of the phospholipids of the adrenal cortex mitochondria was prompted by two observations which led us to suggest that these mitochondria may have unusual phospholipid composition. First, the unique vesicular ultrastructure of these mitochondria raises the question as to whether they have unusual phospholipid or protein compositions. Second, these mitochondria contain, on a molar basis, several times more P-450 than cytochrome oxidase. Since the P-450 in most tissues is associated with microsomes which contain PE and PC but no DG, it was felt that the large amounts of P-450 in the adrenal cortex mitochondria (11) may be accompanied by a disproportional increase in the amounts of PE and PC. The results show that (1) these mitochondria do have

higher molar PE (or PC)/DG (approximately 6) than other tissues (approximately 4), (ii) extracted P-450 preparations have PE and PC but no DG, and (iii) hydrolysis of the PE and PC of the P-450 preparations is accompanied by a parallel decrease in the 11 β-hydroxylase activity. These results suggest that the enrichment of PE and PC in these mitochondria is the result of the presence of the 11 β-hydroxylase complex.

The results (Table II) also demonstrate heterogeneity of P-450 in these mitochondria. Upon pretreatment of the P-450 particles with phospholipase-C, the phospholipid level decreased as the 11 β-hydroxylase activity decreased, whereas the cholesterol desmolase activity increased. These results suggest that there are, at least, two types of P-450 molecules in adrenal cortex mitochondria : one for 11 β-hydroxylase and the other for desmolase. It appears that the former may be associated with phospholipids while the later is not. This may explain why the desmolase is more easily extracted from the mitochondria than the 11 β-hydroxylase; the lipid-associated P-450 molecules may be embedded in the inner membrane. These results are also in accord with the observation of Ungar (3) and of Boyd (4) that an excess of PE or PC inhibits the desmolase activity.

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