ACTION OF THE TRANSFERASE ACTIVITY OF PHOSPHOLIPASE D ON RAT LIVER MITOCHONDRIA

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The action of phospholipase D on rat liver mitochondria in the presence of methanol, glycerol, and ethanolamine has been studied. The phospholipid compositions of the modified and native mitochondria have been determined. Incubation of the mitochondria with phospholipase D led to a considerable decrease in the activities of cytochrome c oxidase and NADH-cytochrome reductase.

A considerable number of enzymes and enzyme systems function only in the presence of phospholipids. Such lipid-dependent enzymes change their activity under the influence both of individual phospholipids (phosphatidylcholine, phosphatidylethanolamine, etc.) and of individual components of the phospholipids (fatty acids, lyso derivatives). The action of different phospholipases is most frequently used to investigate the influence of phospholipids on enzymatic activity [1-3]. It has been established that many enzyme systems of mitochondria are lipid-dependent, and a change in phospholipid composition considerably affects the activities of cytochrome c oxidase, succinate-cytochrome c reductase, and NADH-cytochrome c reductase [4-6]. In all the investigations use was made of hydrolytic phospholipases removing from phospholipid molecules either the polar bases under the action of phospholipases C and D or fatty acids from position 2 of glycerol under the action of phospholipase A_2.

The reactivation by various phospholipids of cytochrome c isolated without lipids showed a clear dependence of the increase of the activity of the enzyme on the addition of many phospholipids, particularly cardiolipin [7]. The aim of our work was to investigate the effect of a change in the phospholipid composition of mitochrondria under the action of the transferase activity of phospholipase D on the cytochrome c oxidase and the NADH-cytochrome c reductase activities of rat liver mitochondria.

The change in the phospholipid composition of the mitochondria under the action on them of phospholipase D in the presence of a donor capable of replacing the alcohol residue of phosphoglycerol is shown in Table 1. The treatment of the mitochondria was carried out in 0.05 M Tris-HCl buffer, pH 7.5, under the conditions most favorable for the retention of the native state of the mitochondria and not the optimum conditions for the action of the phospholipase D. When a suspension of mitochondria was diluted tenfold with 0.05 M Tris-HCl buffer, pH 7.5, the concentrations of lysophosphatidylcholine and of lysophosphatidylethanolamine increased, which is connected with a marked increase in the activity of the endogenous phospholipase A of the mitochondria, the activity of which is fairly high [8].

In the presence of methanol, phospholipase D appreciably changed the phospholipid composition of the motochondria. The amount of phosphatidylcholine fell sharply and the concentration of phosphatidylmethanol rose, this being particularly appreciable under the action of phospholipase D with methanol in the presence of 5 μM of calcium. The presence of glycerol in the incubation medium led to greater hydrolysis of the phosphatidylethanolamine than of the phosphatidylcholine, and the concentration of lysophosphatidylethanolamine rose sharply while the formation of phosphatidylglycerol was slight — 2.8-4.1% of the total amoun of phospholipids. The results of the action of phospholipase D on the phospholipid composition of mitochondria in the presence of ethanolamine are difficult to interpret, since the formation of phosphatidylethanolamine by base substitution is superposed on the hydrolysis of the mitochondrial phosphatidylethanolamine by the endogenous phospholipase, as is confirmed by the high concentration of lysophosphatidylethanolamine, particularly under the action of the enzyme in the presence of calcium.

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TABLE 1. Phospholipid Compositions of Mitochondria under the Action of Phospholipase D (transferase activity) in Tris-HCl Buffer, pH 7.5

Treatment of the mitochondria	Percentages of individual phospholipids ± m*												
	PC	PE	SM	LPC	LPE	PI	PS	PA	DPG	PM	PG	X_1	X_2
Native mitochondria	45,6 ±0,4	37,0 ±0,2	4,78 ±0,2	—	—	3 17 ±0,2	1 6 ±0,1	—	5,93 ±0,3	—	—	1,38 ±0 1	—
Mitochondria in Tris-HCl buffer, pH 7.5	36 1 ±0,3	30,4 ±0,1	6,8 ±0,1	1,75 ±0,25	2,3 ±0,06	—	11,6 ±0 05	—	6,22 ±0,2	—	—	3,9 ±0,06	0 67 ±0.05
Mitochondria + phospholipase D + 10% methanol	34,2 ±0,5	18,7 ±0,5	5,18 ±0.4	4,32 ±0,07	11,2 ±0,1	5,37 ±0 3	3,25 ±0 1	2.5 ±0.2	8,2 ±0,2	3,68 ±0 08	—	3,75 ±0,3	—
Mitochondria + phospholipase D + 10% methanol + 5 μm Ca^{2+}	7.92 ±0,07	30 53 ±0,5	6,56 ±0,3	3,9 ±0,05	8,8 ±0,1	3,9 ±0,1	4,2 ±0,3	7,9 ±0,1	10,2 ±0,09	12.0 ±0,4	—	3,9 ±0.1	—
Mitochondria + phospholidipase D + 10% glycerol	32,1 ±0,8	8,22 ±0,02	6,87 ±0,2	5 2 ±0,2	12,6 ±0,6	5,4 ±0,1	3 48 ±0,5	3,93 ±0,03	10,3 ±0.2	—	2,51 ±0,13	4,75 ±0.2	4 1 ±0 1
Mitochondria + phospholipase D + 10% glycerol + 5 μM Ca^{2+}	23,1 ±0.5	6,97 ±0,07	6,87 ±0 15	6 57 ±0,01	16,6 ±0.0	5,06 ±0,24	3,9 ±0,08	7.58 ±0,01	9.26 ±0,4	—	4,14 ±0,01	5,43 ±0 02	4,47 ±0,11
Mitochondria + phospholipase D + 10% ethanolamine	40.6 ±0,7	28,8 ±0,7	5,66 ±0.54	1,74 ±0,1	5,94 ±0,2	3 3 ±0,3	1,78 ±0,08	1,72 ±0.2	7 05 ±0,2	—	—	3,65 ±0,05	—
Mitochondria + phospholipase D + 10% ethanolamine + 5 μM Ca^{2+}	16.2 ±0,4	21,0 ±0,9	4,35 ±0 4	3,32 ±0,4	26,0 ±1,0	2,52 ±0,3	2,31 ±0,09	7,8 ±0 5	10,8 ±0,15	—	—	5,78 ±0,1	—

*Here and in Table 2: PC — phosphatidylcholine; PE — phosphatidylethanolaime; SM — sphingomyelin; LPC — lysophosphatidylcholine; LPE — lysophosphatidylethanolaime; PI — phosphatidylinositol; PS — phosphatidylserine; PA — phosphatidic acid; DPG — cardiolipin; PM — phosphatidylmethanol; PG — phosphatidylglycerol; X_1 and X_2 — unidentified phospholipids.

We give the results on the activities of cytochrome c oxidase and NADH-cytochrome c reductase in rat liver mitochondria treated with phospholipase D (cytochrome c oxidase: μmole of cytochrome c oxidized/min/mg of protein, NADH-cytochrome c reductase: μmole of cytochrome c reduced/min/mg of protein);

Treatment of the mitochondria	Cytochrome c oxidase	NADH-cytochrome c reductase
0.05 M Tris-HCl buffer, pH 7.5		
Native mitochondria	33.9	98.5
Mitochondria in 0.05 M tris HCl buffer, pH 7.5	26.5	91.9
Mitochondria + phospholipase D + 10% methanol	23.0	97.5
Mitochondria + phospholipase D + 10% methanol + 5 μM Ca^{+}	17.5	73.2
Mitochondria + phospholipase D + 10% glycerol	23.7	
Mitochondria + phospholipase D + 10% glycerol + 5 μM Ca^{2+}	26.9	
0.02 M Acetate buffer, pH 6.0		
Native mitochondria	29.3	75.0
Mitochondria in 0.02 M acetate buffer, pH 6.0	38.2	41.2
Mitochondria + phospholipase D + 10% methanol	29.3	12.0
Mitochondria + phospholipase D + 10% methanol + 5 μM Ca^{2+}	17.7	12.7
Mitochondria + phospholipase D + 10% glycerol	15.2	23.6
Mitochondria + phospholipase D + 10% glycerol + 5 μM Ca^{2+}	18.7	22.1

The activities of the cytochrome c oxidase and the NADH-cytochrome c reductase decreased when the mitochondria were added to 0.05 M Tris-HCl buffer and the conditions were created for the working of the endogenous phospholipases. A change in the phospholipid composition of the mitochondria did not lead to an appreciable loss of activity of the enzymes and this remained, particularly in the presence of glycerol, the same as in the manifestation of the working of the inherent phospholipases A of the mitochondria. However, the appearance in the mitochondria of phosphatidylmethanol, a phospholipid not characteristic of mitochondria, lowered the activity of the cytochrome c oxidase more considerable, while the activity of the NADH-cytochrome c reductase scarcely changed in comparison with a native preparation of mitochondria.

It is known that the activity of cytochrome c oxidase from ox heart mitochondria depends on the amount of phospholipids in the following sequence: phosphatidylethanolamine > cardiolipin > phosphatidylcholine [9]. The treatment of mitochondria with phospholipase D in 0.02 M acetate buffer, pH 6.0, under conditions more favorable for the action of phospholipase D led to a more considerable formation of phosphatidylmethanol in the reaction with methanol and of phosphatidylglycerol in the reaction with glycerol than at pH 7.5 (Table 2). In this case, as well, the amount of lyso derivatives was fairly considerable, particularly that of lysophosphatidylethanolamine, and it reached 21% of the total amount of phospholipids on the reaction of phsopholipase D in the presence of glycerol and calcium. Such marked changes in phospholipid composition strongly affect the activities of cytochrome c oxidase and NADH-cytochrome c reductase. The activity of the NADH-cytochrome c reductase fell sharply, particularly on treatment with phospholipase D in the presence of methanol; the formation of 22-24% of phosphatidylmethanol lowered the activity 3.5-fold in comparison with the activity of the enzyme in mitochondria in acetate buffer. The formation of phosphatidylglycerol and a large amount of lysophosphatidylethanolamine in mitochondria on the reaction of phospholipase D in the presence of glycerol also decreased the activities both of cytochrome c oxidase and of NADH-cytochrome c reductase. The more considerable fall in the activities of the enzymes on incubation with phospholipase D at H 6.0 is due mainly to the greater change in the phospho-

TABLE 2. Phospholipid Compositions of Mitochondria under the Action of Phospholipase D (transferase activity) in 0.02 M Acetate Buffer, pH 6.0

Treatment of the mitochondria	Percentages of the individual phospholipids ± m												
	PC	PE	SM	LPC	LPE	PI	PS	PA	DPG	PM	PG	X_1	X_2
Native mitochondria	45,6 ±0,4	37,06 ± ,25	4,78 ±0,2	— —	— —	3,17 ±0,2	1,6 ±0,06	— —	5,93 ±0,2	— —	— —	1,39 ±0 07	— —
Mitochondria in 0.02 M acetate buffer, pH 6.0	38,8 ±0,5	31,9 ±0,4	5,31 ±0,05	2,36 ±0,06	2,64 ±0,04	— —	11 1 ±0,3	— —	4,93 ±0 13	— —	— —	3,13 ±0,0	0,72 ±0,01
Mitochondria + phospholipase D + 10% methanol	7,2 ±0,6	18,4 ±0,1	7,7 ±0,4	4,6 ±0,1	9,6 ±0,4	3,5 ±0,3	4,4 ±0,5	5 8 ±0,02	9 0 ±1 2	24,2 ±0,4	— —	5,2 ±0,2	— —
Mitochondria + phospholipase D + 10% methanol + 5 μM Ca^{2+}	2,9 ±0,0	24 7 ±0,7	7,8 ±0,03	2,6 ±0,24	3,7 ±0,04	2,5 ±0,01	5,7 ±0,6	14,0 ±0,6	9,5 ±0,2	22 5 ±0,5	— —	3,7 ±0,07	— —
Mitochondria + phospholipase D + 10% glycerol	27,0 ±0,1	12,8 ±0,2	6,8 ±0,2	5,1 ±0,07	17,4 ±0,2	2,3 ±0,2	2 6 ±0,08	4,1 ±0,08	8 ' ±0,2	— —	7,8 ±0,15	3 0 ±0 2	2,6 ±0,06
Mitochondria + phospholipase D + 10% glycerol + 5 μM Ca^{2+}	21,78 ±0,3	9,1 ±0,1	7,3 ±0,2	5,0 ±0,2	21,0 ±1,1	2,6 ±0,4	3,6 ±0,2	5,3 ±0,1	9,3 ±0,7	— —	5,9 ±0,04	4,9 ±0,2	3,6 ±0,03
Mitochondria + phospholipase D + 10% ethanolamine	32,2 ±0,7	29,7 ±0,7	4,7 ±0,05	1,9 ±0,1	6,8 ±0,05	5,4 ±0,2	3,08 ±0,1	4,0 ±0,4	7,8 ±0,1	— —	— —	4,3 ±0,1	— —

lipid composition of the mitochondria. Thus, the activity of cytochrome c oxidase, which is increased in 0.02 M acetate buffer, pH 6.0, without the addition of phospholipase D as compared with the native mitochondria, fell when the mitochondria were treated with phospholipase D in the presence of 10% of glycerol. The sensitivity of NADH-cytochrome c reductase to a change in the lipid composition was higher, its activity in acetate buffer amounting to half of the activity in the native mitochondria and 1/3 of the initial activity when the mitochondria were treated with phospholipase D in acetate buffer in the presence of glycerol and calcium.

When cytochrome c oxidase from ox heart mitochondria that had been freed from lipids was reactivated with synthetic phospholipids it was found [7] that the greatest activating capacity was possessed by lysophosphatidylcholine with oleic or palmitic fatty acids, by dipalmitoylphosphorylcholine, and by lysooleoylphosphatidic acid. The heterogeneity of the phospholipid composition in our experiments did not enable us to find such a clear situation with regard to activation or inhibition by individual phospholipids of the activities of the enzymes of the mitochondria, but having analyzed our results we can make the following observations:

1) the dilution of a native preparation of mitochondria with Tris-HCl buffer, pH 7.5, or acetate buffer, pH 6.0, considerably changes the phospholipid composition because of the activation of endogenous phospholipases;

2) the incubation of mitochondria with phospholipase D in the presence of methanol, glycerol, and ethanolamine causes a marked change in the phospholipid composition of the mitochondria, leading to the formation of phospholipids foreign to the mitochondria — phosphatodylmethanol, phosphatidylglycerol, and high concentrations of lysophosphatidylethanolamine; and

3) the incubation of the mitochondria with phospholipase D considerably changes the activities of the cytochrome c oxidase and NADH-cytochrome c reductase. A greater change in the phospholipid composition corresponds to a more considerable decrease in the activity of the mitochondrial enzymes.

The method of changing the phospholipid composition of mitochondria that we have used is one more example of the use of transferase activity of phospholipase D for modifying membrane phospholipids. Gercken and Witt [10] have used phospholipase D (transferase activity) to modify the phospholipid composition of erthyrocyte ghosts and have established a dependence of the change in the activity of ATPase on the phospholipid composition of membranes.

EXPERIMENTAL

The rat liver mitochondria were isolated by the method of Johnson and Lardy [11] and phospholipase D was isolated from cabbage leaves by the method of Davidson and Long [12]. The cytochrome c oxidase activity was determined by the method of Wharton and Tzagoloff [13] and the NADH-cytochrome c reductase activity by the Fleishers' method [14]. The quantitative analysis of the phospholipids was carried out after two-dimensional chromatography by Vaskovsky's method [15].

Treatment of the Membranes with Phospholipase D. A mixture was made of 0.1 ml of a suspension of mitochondria (7.2 mg of protein/ml) in 0.05 M Tris-HCl—0.25 M sucrose—0.001 M EDTA solution; 0.16 ml of a solution of phospholipase D in 0.05 M Tris-HCl buffer, pH 7.5, or in 0.02 M acetate buffer, pH 6.0; 0.24 ml of the corresponding buffer; and 10% of the total volume of methanol, glycerol, or ethanolamine. Incubation was carried out at 23°C for 30 min. The mitochondria were separated by centrifugation at 10,000 rpm for 10 min, the deposit was washed with 0.05 M Tris-HCl—0.25 M sucrose—0.001 M EDTA solution, and was recentrifuged under the same conditions, and the mitochondria were then suspended in 1.0 ml of solution for the isolation and storage of the mitochondria. The phospholipids were extracted with chloroform—ethanol (2:1) and aliquots of the chloroform layer were deposited on a chromatographic plate. Protein was determined by Lowry's method [16] using serum albumin as standard.

SUMMARY

The endogenous phospholipases of mitochondria are activated in tris-HCl or acetate buffer, which leads to the formation of 2-3% of lyso derivatives of phospholipids. The incuba-

tion of mitochondria with phospholipase D in the presence of methanol, glycerol, or ethanolamine changes the phospholipid composition of the mitochondria and leads to the formation of phosphatidylmethanol, phosphatidylglycerol, and high concentrations of lysophosphatidylethanolamine. The incubation of mitochondria with phospholipase D causes mainly a considerable decrease in the activities of cytochrome c oxidase and NADH-cytochrome c reductase.

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ISOLATION AND CHARACTERIZATION OF AN L-AMINO ACID ACYLASE FROM *Aspergillus oryzae*

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A scheme of isolating a highly purified L-amino acylase from *Aspergillus oryzae* is described which excludes extraction of the enzyme from the preparation "Amilorizin," fractionation with ethanol, chromatography on DEAE-cellulose, and gel filtration through Sephadex G-200 and Bio-Gel P-300. The enzyme, as purified 1240-fold, has a molecular weight of 118,000, apparently consists of two subunits with a molecular weight of 60,000, is stable in the pH range of 7-10 and has an optimum pH of 8.9 and a pI of 4.0. Its amino acid composition has been determined and its substrate specificity has been studied. The acylase is a metalloenzyme: Co^{2+} ions in concentrations of 10^{-4}-$5 \cdot 10^{-5}$ M increase the rate of hydrolysis of N-acetyl-L-amino acids three- to fourfold. It shows differences in its molecular and functional properties from acylase I obtained from porcine kidney.

L-Amino acid acylase (E.C. 3.5.1.14) hydrolyses the amide bonds of N-acetyl-L-amino acids. This enzyme has been detected in various microorganisms — fungi, bacteria, and yeasts [1, 2]. The acylases of microscopic fungi are used for the isolation of L-amino acids from the racemates obtained by chemical synthesis. Unpurified or partially purified preparations of *Aspergillus oryzae* are used for this purpose [3-5]. However, hitherto there has been no information on the properties of pure acylases of microscopic fungi. The purification of fungal acylases is made difficult by the fact that the initial preparations contain a multiplicity of other proteins, enzymes, and pigments. For example, a surface culture of

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