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THE ROLE OF LIPID-PROTEIN INTERACTIONS IN NADH-CYTOCHROME *c* REDUCTASE (ROTENONE-INSENSITIVE) OF RAT LIVER MITOCHONDRIA

F. FEO, R.A. CANUTO, R. GARCEA and O. BROSSA

Istituto di Patologia generale dell'Università di Torino, Cso Raffaello 30, 10125, Torino, and Cattedra di Patologia generale II dell'Università di Sassari, via Padre Manzella 4, 07100, Sassari (Italy)

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Summary

The phospholipid depletion of rat liver mitochondria, induced by acetone-extraction or by digestion with phospholipase A_2 or phospholipase C, greatly inhibited the activity of NADH-cytochrome *c* reductase (rotenone-insensitive). A great decrease of the reductase activity also occurred in isolated outer mitochondrial membranes after incubation with phospholipase A_2. The enzyme activity was almost completely restored by the addition of a mixture of mitochondrial phospholipids to either lipid-deficient mitochondria, or lipid-deficient outer membranes. The individual phospholipids present in the outer mitochondrial membrane induced little or no stimulation of the reductase activity. Egg phosphatidylcholine was the most active phospholipid, but dipalmitoyl phosphatidylcholine was almost ineffective. The lipid depletion of mitochondria resulted in the disappearance of the non-linear Arrhenius plot which characterized the native reductase activity. A non-linear plot almost identical to that of the native enzyme was shown by the enzyme reconstituted with mitochondrial phospholipids. Triton X-100, Tween 80 or sodium deoxycholate induced only a small activation of NADH-cytochrome *c* reductase (rotenone-insensitive) in lipid-deficient mitochondria. The addition of cholesterol to extracted mitochondrial phospholipids at a 1 : 1 molar ratio inhibited the reactivation of NADH-cytochrome *c* reductase (rotenone-insensitive) but not the binding of phospholipids to lipid-deficient mitochondria or lipid-deficient outer membranes.

These results show that NADH-cytochrome *c* reductase (rotenone-insensitive) of the outer mitochondrial membrane requires phospholipids for its activity. A mixture of phospholipids accomplishes this requirement better than individual phospholipids or detergents. It also seems that the membrane fluidity may influence the reductase activity.

Introduction

Interactions between lipid and protein have been shown to regulate several transport and enzyme activities in membranes [1—3]. They induce, at the molecular level, modifications of protein conformation [4] as well as restriction of the motion of phospholipids [5—7]. The topography, functions and relationships with lipids have been largely investigated for enzymes of ergastoplasmic reticulum, inner mitochondrial membrane and plasma membrane (see refs. 2 and 3 for reviews). Relatively little attention has been dedicated to the outer membrane of mitochondria. To our knowledge only one study dealing with the lipid-dependence of enzymes of the outer mitochondrial membrane has been published so far [8]. Dependence on membrane lipids was demonstrated for kynurenine hydroxylase activity, while it was not found for monoamine oxidase [8].

Previous work from our laboratory [7,9] has shown a break in the Arrhenius plot for NADH-cytochrome *c* reductase (rotenone-insensitive) of rat liver outer mitochrondrial membrane. By contrast, no discontinuity in the activation energy was found for this enzyme in mitochondria from Yoshida hepatoma AH-130, whose outer membrane contains four times as much cholesterol as the outer membrane of liver mitochondria [10].

In the present report the interrelationships between NADH-cytochrome *c* reductase (rotenone-insensitive) and lipids have been further investigated in rat liver mitochondria. Evidence of lipids dependence of this enzyme is presented and the effect of cholesterol on the enzyme activity is illustrated.

Materials and Methods

Fractionation procedure. The isolation of rat liver mitochondria was described in a previous paper [10]. The particles were washed five times and then suspended in 0.25 M sucrose at concentrations of 10—25 mg protein/ml. Outer mitochondrial membranes were isolated and purified according to the procedure of Sottocasa et al. [11] as modified by Jones and Jones [12]. In brief, mitochondria (7—10 mg protein/ml) were dispersed in 10 mM sodium phosphate buffer (pH 7.4). After 10 min incubation at 0°C, KCl, ATP and $MgCl_2$ were added to a final concentration of 0.480, 0.005 and 0.005 M, respectively [12]. After an additional 5 min incubation, the suspensions were subjected to 10 s of sonic oscillation with the use (at 60% of its maximal output) of a Biosonik III sonifier (Bronwill Scientific, Rochester, N.Y.) provided with an intermediate tip. 20 ml of the sonicated suspensions (about 50 mg of protein) were layered on top of 18.5 ml of 1.18 M sucrose, and centrifuged for 3.5 h at 95 000 × *g* in a Spinco SW-27 rotor. The interface band (outer membranes) was collected, diluted with 0.25 M sucrose and centrifuged 12 min at 13 500 × *g*. The pellet was suspended in 0.25 M sucrose at a concentration of 20 mg protein/ml. The purity of the outer membrane preparations was routinely evaluated by determining cytochrome *c* oxidase and NADPH-cytochrome *c* reductase activities as markers for inner mitochondrial membrane and microsomes, respectively. From the specific activities of the marker enzymes in the various fractions, concentrations of inner membranes in the

outer membrane fractions ranging from 3.0 to 4.5% were calculated. The concentrations of microsomes in the outer membrane fraction ranged from 0 to 4.5%.

Prepration of lipid-deficient fractions. A method patterned on the procedure of Fleischer and Fleischer [13] was used to prepare lipid-deficient mitochondria by extraction with acetone/water. 1 ml mitochondria (25 mg protein), suspended in 0.25 M sucrose, was added to 24 ml acetone/water (22.5 ml dry acetone and 1.5 ml distilled water) and allowed to stand 15 min at 0°C with occasional swirling. The mixture was centrifuged at 5000 × *g* for 2 min. The pellet was washed twice in 0.88 M sucrose/0.01 M Tris/Cl buffer (pH 7.4) and finally suspended in 2.5 ml of the same medium.

In order to perform the digestion with phospholipase of membrane phospholipids, whole mitochondria or isolated outer membranes (10 mg protein/ml) were suspended in 2—10 ml of a reaction mixture containing 0.1 M sucrose, $0.5 \cdot 10^{-4}$ M glycyl glycine buffer (pH 7.5), 0.004 M $CaCl_2$ and 1% bovine serum albumin (defatted). The reaction mixture was allowed to equilibrate 5 min at 37°C. The reaction was started by the addition of 200 μg phospholipase C (from *Bacillus cereus*, Boehringer, Mannheim, G.F.R.) or 5 μg phospholipase A_2 (from *Vipera Russelli*, Sigma Chemical Co., St. Louis, Mo., U.S.A.) per mg mitochondrial protein. After different times of incubation at 37°C, EDTA was added to a concentration of 0.01 M. The suspensions were rapidly cooled, centrifuged for 18 min at 114 000 × *g* and washed three times in a medium containing 0.25 M sucrose, 0.001 M EDTA, 0.05 M glycyl glycine buffer (pH 7.5) and 1% bovine serum albumin (defatted). After one more washing in 0.88 M sucrose/0.01 M Tris/Cl, the pellets were suspended in the latter medium to yield a protein concentration of approx. 10 mg per ml.

Dispersion of lipids. Mitochondrial lipids were extracted under nitrogen as described in a previous publication [14]. 75 mg of phospholipids from different sources, dissolved in chloroform, were evaporated under nitrogen in a test tube and the last traces of solvent removed in vacuo. 8 ml of a medium containing 0.02 M Tris/acetate (pH 8) and 0.001 M EDTA [13] were added to the dry films. The lipids were dispersed by vortexing the stoppered tubes for 15 min above the transition temperatures of the lipids. The dispersions were then submitted to sonic oscillation for 10 min at 10°C, under nitrogen bubbling, with the use of a Biosonik III sonifier at its maximal output. Care was taken to avoid foaming. The suspensions were centrifuged for 18 min at 114 000 × *g*. Clear supernatants were taken as the source of lipid vesicles. Chromatographically pure phosphatidylcholine and phosphatidylethanolamine from egg, and phosphatidylinositol from pig brain were purchased from Koch-Light Laboratory (Colnbrook, U.K.). Dipalmitoyl phosphatidylcholine was purchased from Sigma Chemical Co.

Rebinding of lipids to lipid-deficient preparations. The lipid rebinding was performed by mixing lipid-deficient mitochondria or lipid-deficient outer membranes, suspended in 0.88 M sucrose/0.01 M Tris/Cl buffer (pH 7.4), with equal amounts of lipid vesicle suspensions (10 μmol phospholipid/ml). After 30 min incubation at 30°C, the suspensions were centrifuged for 10 min at 47 000 × *g* and washed once in cold sucrose/Tris medium. The pellets were suspended in the same medium to reach a concentration of 10 mg of protein

per ml. Alternatively, 0.1-ml aliquots of lipid-deficient mitochondria (15—35 μg protein) or lipid-deficient outer membranes (8 μg protein) were added to 1 ml of reaction mixture for the determination of NADH-cytochrome *c* reductase (rotenone-insensitive). After 10 min incubation at 30°C (unless otherwise stated) with various amounts of lipid dispersions, the monitoring of the enzymatic activity was initiated with NADH.

Enzyme assays. The determination of cytochrome *c* oxidase activity was performed according to De Duve et al. [15]. NADH- and NADPH-cytochrome *c* reductase activities were measured spectrophotometrically at 30°C by following the reduction of cytochrome *c* at 550 nm. The reaction mixture contained in 1 ml: $3 \cdot 10^{-4}$ M NADH or NADPH/$1 \cdot 10^{-4}$ M cytochrome *c*/$3 \cdot 10^{-4}$ M KCN/$8 \cdot 10^{-2}$ M potassium phosphate buffer (pH 7.4) and, when NADH was the substrate, $4 \cdot 10^{-6}$ M rotenone. The reaction was started by the addition of NADH or NADPH.

Analytical determinations. The procedures for lipid extraction, purification and determination have already been described [14]. Proteins were determined by a biuret procedure [16].

Results

Effect of acetone extraction on enzyme activity. The extraction of mitochondria with 90% acetone was shown to inactivate several enzymes linked to the mitochondrial inner membrane [17,18]. The inactivation of the outer membrane enzyme kynurenine hydroxylase was also described [8]. Solvent extraction resulted in a great decrease of mitochondrial phospholipids [13]. As illustrated in Fig. 1, the treatment of mitochondria with 90% acetone induced an 87% decrease of NADH-cytochrome *c* reductase (rotenone-insensitive). This treatment also caused approximately a 74% loss of the phospholipid content (not shown). The requirement for phospholipids of NADH-cytochrome *c* reductase (rotenone-insensitive) is shown by the experiment described in Fig. 1. Upon addition of increasing amounts of mitochondrial phospholipids to lipid-deficient mitochondria, about 80% of the control enzyme activity was restored.

Effect of digestion with phospholipases on enzyme activity. Further evidence for a phospholipid dependence of NADH-cytochrome *c* reductase (rotenone-insensitive) emerged from experiments with phospholipase C and phospholipase A_2. By changing the time of incubation of mitochondria with each of the two enzymes, different degrees of interruption of lipid-protein interactions may by obtained. This is seen from data in Table I. The decrease of the reductase activity is in good correlation with the loss of mitochondrial phospholipids. In whole mitochondria incubated with phospholipase C, the enzyme inhibition was 38% and 72% when the extent of lipid hydrolysis reached 49% and 84%, respectively. With phospholipase A_2, the enzyme inhibition ranged between 52 and 88% and the extent of lipid hydrolysis ranged between 50 and 92%. Similar results were obtained with isolated outer membranes; the treatment for different times with phospholipase A_2 resulted in enzyme inhibition ranging from 43 to 88%, while the phospholipid digestion ranged from 52 to 86%. EDTA completely prevented the inhibitory effect of

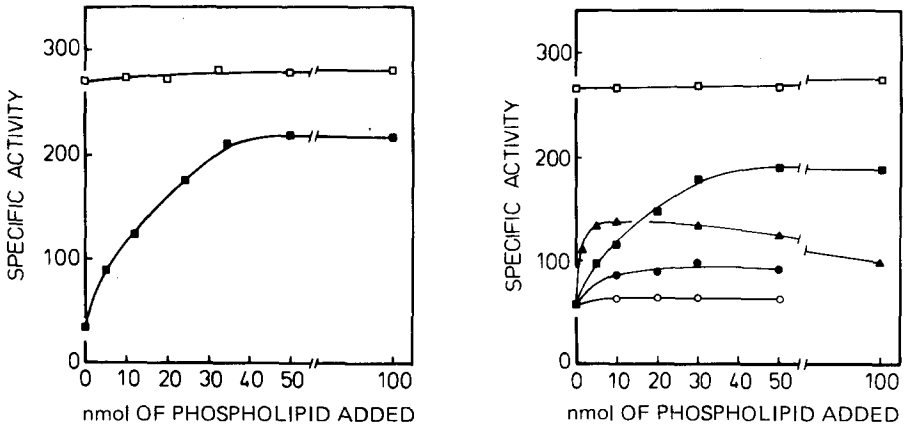

Fig. 1. Activation by phospholipids of NADH-cytochrome *c* reductase (rotenone-insensitive) in acetone-extracted mitochondria. Lipid-deficient mitochondria were prepared by solvent extraction with 90% acetone as described under Materials and Methods. Untreated mitochondria were used as the source of native enzyme. 0.1 ml aliquots of either extracted or unextracted mitochondria (15 μg protein), suspended in 0.88 M sucrose/0.01 M Tris/Cl (pH 7.4), were added to 1 ml of reaction mixture for the determination of the enzyme activity. 0.1 ml of dispersions of mitochondrial lipids, containing the indicated amounts of phospholipids, were added directly to the assay cuvettes and the suspensions were incubated 10 min at 30°C prior to the start of the reaction with NADH. Specific activity, nmol cytochrome *c* reduced/min per mg protein. □, acetone-extracted mitochondria; ■, unextracted mitochondria.

Fig. 2. Activation by phospholipids of NADH-cytochrome *c* reductase (rotenone-insensitive) of mitochondria submitted to phospholipase A_2 digestion. Mitochondria were incubated for 30 min at 37°C with 5 μg phospholipase A_2/mg protein. The reaction was arrested by addition of EDTA to a concentration of 0.01 M. The mitochondria were then washed three times with serum albumin in sucrose/glycyl glycine/EDTA buffer and finally suspended in 0.88 M sucrose/0.01 M Tris/Cl (pH 7.4). Untreated mitochondria were used as the source of native enzyme. 0.1 ml aliquots of different phospholipid dispersions, containing the indicated amounts of phospholipids, were added to the assay cuvettes. The conditions for incubation of intact or lipid-deficient mitochondria with phospholipids and for reductase determination were the same as in Fig. 1. The phospholipid tested were: □, ■, mixed mitochondrial phospholipids; ▲, egg phosphatidylcholine; ●, egg phosphatidylethanolamine; ○, pig brain phosphatidylinositol. □, enzyme activity of untreated mitochondria. Specific activity, nmol of cytochrome *c* reduced/min per mg protein.

phospholipases. This indicates that the enzyme inhibition could not be due to possible contamination of the phospholipase preparations by proteolytic enzymes.

Reconstitution experiments with lipid-deficient mitochondria prepared by incubation with phospholipase A_2, showed that a mixture of mitochondrial phospholipids restored about 70% of the initial activity of NADH-cytochrome *c* reductase (rotenone-insensitive), (Fig. 2). Among the individual phospholipids, approximately 53% of initial activity was restored by phosphatidylcholine. At high concentrations this phospholipid was inhibitory. Phosphatidylethanolamine induced a small restoration of initial activity (approx. 30%), while phosphatidylinositol was without effect. The activation by phospholipids of NADH-cytochrome *c* reductase (rotenone-insensitive) in isolated outer membranes is shown in Fig. 3. It can be seen that after incubation with phospholipase A_2 the enzyme activity was decreased to 12% of the control value. Addition of graded amounts of mitochondrial phospholipids restored 75% of the native enzyme activity. As in the case of whole mitochondria, some

TABLE I

EFFECT OF PHOSPHOLIPASE TREATMENT ON NADH-CYTOCHROME *c* REDUCTASE (ROTENONE-INTENSITIVE) ACTIVITY AND PHOSPHOLIPID CONTENT OF WHOLE MITOCHONDRIA AND ISOLATED OUTER MEMBRANES

The treatment of mitochondria and outer membranes with phospholipases is described in Materials and Methods. The reaction was started by the addition of phospholipases and stopped, after the times indicated, by the addition of EDTA to a final concentration of 0.01 M. When indicated, EDTA was added before phospholipases. Temperature, 37°C. After incubation, the mitochondria or the outer membranes were washed three times with serum alnumin in sucrose/glycyl glycine/EDTA buffer (cf. Materials and Methods). Samples of lipid-deficient mitochondria (15 μg protein) or lipid-deficient outer membranes (8 μg protein) were assayed at 30°C in 1 ml of reaction mixture for the determination of the reductase activity. The reaction was started with NADH. Specific activity nmol of cytochrome *c* reduced/min per mg of protein. Data are mean values ± S.D.

Treatment and fractions	Time of incubation (min)	Number of experiments	Inhibition of enzyme activity (%)	Decrease of phospholipid content (%)
Whole mitochondria				
Phospholipase C	15	3	38 ± 8	49 ± 3
Phospholipase C	30	3	72 ± 12	84 ± 12
Phospholipase C + EDTA	30	2	2	6
Phospholipase A_2	15	3	52 ± 6	50 ± 9
Phospholipase A_2	30	8	92 ± 9	78 ± 6
Phospholipase A_2	45	4	88 ± 7	92 ± 18
Phospholipase A_2 + EDTA	45	2	3	4
Outer membranes				
Phospholipase A_2	0.25	2	43	52
Phospholipase A_2	1	2	70	73
Phospholipase A_2	2	9	88 ± 13	86 ± 9
Phospholipase A_2	5	5	82 ± 12	86 ± 15
Phospholipase A_2 + EDTA	5	2	6	8

restoration of the native enzyme activity was obtained with phosphatidylcholine and with phosphatidylethanolamine. The maximal restoration, attained with 10 nmol of lipid per ml, was 40% for phosphatidylcholine and 27% for phosphatidylethanolamine. Once more phosphatidylinositol was without effect.

In some experiments with lipid-deficient mitochondria, dipalmitoyl phosphatidylcholine was substituted for egg phosphatidylcholine. This resulted in an almost complete loss of stimulation on the reductase activity. In the presence of 5—50 nmol dipalmitoyl phosphatidylcholine per ml reaction mixture, specific activities of 88.51 to 95.25 nmol of cytochrome *c* reduced/min per mg of protein were recorded. The specific activity in control mitochondria was 254.27 (S.D. ± 9.74).

Arrhenius plots of lipid-deficient and reconstituted enzyme. It has been suggested that the lipid microenvironment of membrane-bound enzymes is involved in the regulation of enzymatic activities [7,19,20]. The formation of different lipid clusters in some functional areas of membranes seems to explain differences in the activity transitions of enzymes [7,19,20]. It should be expected that if the lipid microenvironment changed during removal and binding of lipids, the activity transition of NADH-cytochrome *c* reductase (rotenone-insensitive) would become modified.

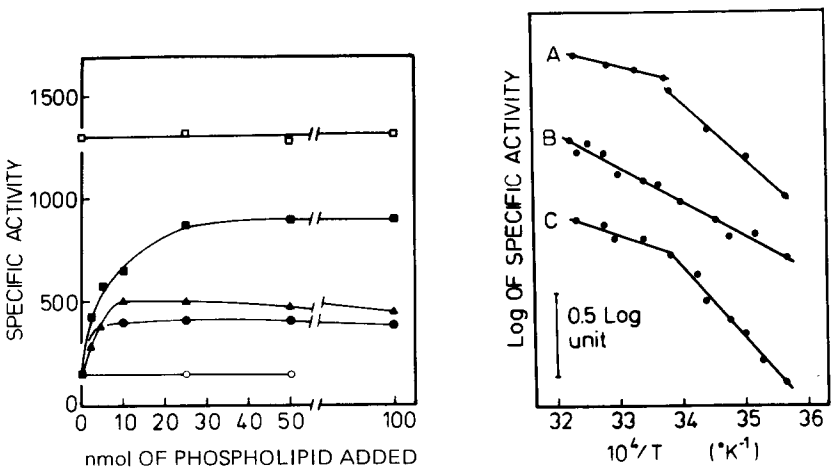

Fig. 3. Activation by phospholipids of NADH-cytochrome *c* reductase (rotenone-insensitive) of isolated outer mitochondrial membranes submitted to phospholipase A_2 digestion. Outer membranes were incubated 2 min at 37°C with 5 μg of phospholipase A_2 per mg protein. After the arrest of the reaction by 0.01 M EDTA, the membranes were washed three times with serum albumin in sucrose/glycyl glycine/EDTA buffer and suspended in 0.88 M sucrose/0.01 M Tris/Cl (pH 7.4). 0.1 ml aliquots of lipid-deficient or untreated membranes (8 μg protein) were added to 1 ml of reaction mixture for the determination of reductase activity. The indicated amounts of phospholipids were added to the assay cuvettes in 0.1 ml aliquots. The incubation of outer membranes with phospholipids and the reductase determination were performed as in Fig. 1. The phospholipids tested were: □, ■, mixed mitochondrial phospholipids; ▲, egg phosphatidylcholine; ●, egg phosphatidylethanolamine; ○, pig brain phosphatidylinositol. □, enzyme activity of undigested membranes. Specific activity, nmol cytochrome *c* reduced/min per mg protein.

Fig. 4. Effect of lipid depletion and lipid rebinding on the Arrhenius profiles of NADH-cytochrome *c* reductase (rotenone-insensitive). Lipid-deficient mitochondria were prepared by incubation for 30 min at 37°C with 5 μg phospholipase A_2/mg protein. 0.1 ml aliquots of lipid-deficient or untreated mitochondria (15—35 μg protein) were added to 1 ml of assay medium for the determination of the reductase activity. They were incubated for 10 min, at the indicated temperatures, prior to the start of the reaction with NADH. When indicated, mitochondrial phospholipids were added to the assay cuvettes to a final concentration of 3 nmol/mg protein. The enzymatic activity was measured spectrophotometrically at temperatures ranging from 6 to 31°C by using a thermostatic cell. The temperatures in the assay medium were controlled by a thermocouple. Specific activity, nmol cytochrome *c* reduced/min per mg protein. A, untreated mitochondria; B, lipid-deficient mitochondria; C, reconstituted mitochondria.

Fig. 4 shows that with intact mitochondria, a non-linear Arrhenius plot was found for reductase with a break at 21°C and with apparent activation energies of about 8.02 and 18.2 kcal/mol. In mitochondria subjected to phospholipase A_2 digestion, no discontinuity in the Arrhenius plot was observed in the range of tested temperatures. The apparent activation energy was 13.6 kcal/mol. The reconstituted enzyme highly resembled the native enzyme with respect to its response to temperature changes. The Arrhenius plot was non-linear with a break at 21.4°C and apparent activation energies of 9.00 and 21.52 kcal/mol. This could indicate that the binding of extracted mitochondrial phospholipids to the active proteins can re-establish a lipid microenvironment having a physical state similar to that in the native outer membrane.

Effect of detergents. Control experiments were performed in order to determine whether the stimulatory effect of phospholipids on NADH-cyto-

TABLE II

EFFECT OF DETERGENTS ON THE RESTORATION OF NADH-CYTOCHROME *c* REDUCTASE (ROTENONE-INSENSITIVE) ACTIVITY IN LIPID-DEFICIENT MITOCHONDRIA

The mitochondria were incubated for 30 min at 37°C with % μg phospholipase A_2 per mg protein. To the lipid-deficient preparations (20 μg protein), suspended in 1 ml of reaction mixture for the determination of the reductase activity, 0.1 ml aliquots of the detergents were added. After incubation for 2 min at 30°C, the enzymatic activity was initiated with NADH. The final concentrations of the detergents (mg protein) were 1.02, 0.83 and 2 for Triton X-100, Tween 80 and sodium deoxycholate, respectively. These concentrations were required to achieve the maximal activity with each detergent. Specific activity, nmol cytochrome *c* reduced/min per mg of protein. Data are mean values of two experiments.

Additions and fractions	Specific activity	Percent
Intact mitochondria		
None	256.21	
Triton X-100	276.02	100
Lipid-deficient mitochondria		
None	62.03	23
Triton X-100	108.13	39
Tween 80	84.58	30
Sodium deoxycholate	100.03	36

chrome *c* reductase (rotenone-insensitive) depended on the surface-active properties of the lipid molecules.

As shown in Table II, only a partial restoration of reductase activity by the addition of detergents to lipid-deficient mitochondria was found. The concentrations given in the Table were calculated from titration curves at which the maximal enzyme activation was attained. Triton X-100 was most effective; however, the maximal activity restored by this detergent was only 39% of that obtained upon addition of Triton X-100 to intact mitochondria and 55% of that attained with extracted phospholipids in lipid-deficient mitochondria (cf. Fig. 2).

Effect of cholesterol on the enzyme activity. Sterols exert a regulatory role on different membrane-bound enzymes [7,21,24]. Aithal et al. [8] observed that kynurenine hydroxylase of yeast mitochondria is activated by increasing the ergosterol content of the organelles. However, sterol contents higher than 27 μg/mg protein induced a decrease of the enzymatic activity. The outer membrane of rat liver mitochondria contains relatively large amounts of cholesterol [25]. Therefore, it was interesting to investigate the effect of cholesterol on the activation of NADH-cytochrome *c* reductase (rotenone-insensitive) by the phospholipids. Data in Table III demonstrate that cholesterol at a 1 : 1 molar ratio with extracted phospholipids almost completely prevents the activation of the enzyme in mitochondria treated with either acetone/water or phospholipase A_2. The same ratio of cholesterol to phospholipids also prevented the enzyme activation in isolated outer membranes treated with phospholipase A_2.

The phospholipid extracted from liver mitochondria contains greater amounts of unsaturated than saturated acyl chains [9,26,27]. The incorporation of cholesterol between phospholipid molecules containing unsaturated paraffin chains causes some membrane areas to be less fluid [28]. Therefore,

TABLE III

EFFECT OF CHOLESTEROL ON THE ACTIVATION OF NADH-CYTOCHROME *c* (ROTENONE-INSENSITIVE) BY PHOSPHOLIPIDS

The extraction of whole mitochondria with 90% acetone, and the digestion of whole mitochondria and isolated outer membranes with 5 μg phospholipase A_2/mg protein are described under Materials and Methods. The incubation times with phospholipase were 30 min for mitochondria and 5 min for outer membranes. After three washings with serum albumin in sucrose/glycyl glycine/EDTA buffer, the fraction were suspended in 0.88 M sucrose/0.01 M Tris/Cl (pH 7.4). 0.1 ml aliquots of mitochondria (15 μg protein) or outer membranes (8 μg protein) were added to 1 ml of reaction mixture for the determination of the reductase activity. Extracted mitochondrial phospholipids were added to the reaction mixture to a final concentration of 50 nmol per ml. After 10 min incubation at 30°C, the reaction was started with NADH. When indicated, cholesterol and phospholipids were mixed at 1 : 1 molar ratio. Data are mean values ± S.D. Specific activity, nmol of cytochrome *c* reduced/min per mg protein.

Treatment and fractions	Additions	Number of experiments	Specific activity
Whole mitochondria			
None	None	8	256.91 ± 12.81
None	Extracted mitochondrial phospholipid	2	282.10
None	Extracted mitochondrial phospholipid/cholesterol	2	274.62
Acetone/water	None	4	36.17 ± 6.56
Acetone/water	Extracted mitochondrial phospholipid	4	222.26 ± 15.10
Acetone/water	Extracted mitochondrial phospholipid/cholesterol	3	55.50 ± 8.61
Phospholipase A_2	None	3	79.35 ± 16.43
Phospholipase A_2	Extracted mitochondrial phospholipid	3	177.24 ± 24.76
Phospholipase A_2	Extracted mitochondrial phospholipid/cholesterol	3	99.19 ± 12.06
Outer membranes			
None	None	12	1264 ± 96
None	Extracted mitochondrial phospholipid	2	1292
None	Extracted mitochondrial phospholipid/cholesterol	2	1176
Phospholipase A_2	None	9	127.23 ± 20.02
Phospholipase A_2	Extracted mitochondrial phospholipid	8	880.61 ± 76.41
Phospholipase A_2	Extracted mitochondrial phospholipid/cholesterol	2	164.06

the inhibitory effect of cholesterol on enzyme reactivation could be related to a failure of lipid-deficient membranes to bind cholesterol-containing vesicles, rather than being related to a modification of the enzyme-lipid interaction. Data in Table IV show that the increase in the phospholipid content was practically the same when the lipid-deficient fractions were incubated with phospholipid vesicles with or without cholesterol. In both instances lipid rebinding reached 70—73% of the initial phospholipid content in whole mitochondria, and 59—61% in the outer membranes. It also appears that large modifications of the mitochondrial cholesterol to phospholipid molar ratio took place when whole mitochondria, as well as outer membranes, were subjected to lipid depletion or lipid rebinding. The cholesterol content per mg protein apparently

TABLE IV

VARIATIONS OF THE CHOLESTEROL AND PHOSPHOLIPID CONTENTS AFTER REMOVAL AND REBINDING OF LIPIDS IN WHOLE MITOCHONDRIA AND ISOLATED OUTER MEMBRANES

Whole mitochondria or isolated outer membranes were incubated at 37°C with phospholipase A_2 (5 μg/mg protein) for 30 and 5 min, respectively. The reaction was arrested by the addition of EDTA to a concentration of 0.01 M. The fractions were washed three times with serum ablumin in sucrose/glycyl glycine/EDTA buffer and finally suspended in 0.88 M sucrose/0.01 M Tris/Cl (pH 7.4). 2—3 ml aliquots of the suspensions (20—30 mg protein) were mixed with equal amounts of lipid vesicle suspensions (3 μmol phospholipid/mg protein) and incubated for 30 min at 30°C. The fractions were then washed and collected as described under Materials and Methods and used for lipid determinations. When indicated, the cholesterol and phospholipids were mixed at 1 : 1 molar ratio. Data are mean values ± S.D. Molecular weight of cholesterol, 387; phospholipid, 700.

Treatment and fractions	Additions	Number of experiments	Phospholipid (mg/mg protein)	Cholesterol (μg/mg protein)	Molar ratio (cholesterol : phospholipid)
Whole mitochondria					
None	None	6	0.184 ± 0.020	3.12 ± 0.91	1 : 32.9
Phospholipase A_2	None	6	0.027 ± 0.002	3.87 ± 1.36	1 : 3.8
Phospholipase A_2	Extracted mitochondrial phospholipids	6	0.135 ± 0.031	5.13 ± 2.54	1 : 12.0
Phospholipase A_2	Extracted mitochondrial phospholipids/ cholesterol	6	0.129 ± 0.023	45.40 ± 9.66	1 : 1.6
Outer membranes					
None	None	13	0.488 ± 0.038	22.57 ± 3.11	1 : 12.0
Phospholipase A_2	None	9	0.043 ± 0.006	19.39 ± 2.26	1 : 1.2
Phospholipase A_2	Extracted mitochondrial phospholipids	2	0.300	20.16	1 : 9.6
Phospholipase A_2	Extracted mitochondrial phospholipid/ cholesterol	2	0.286	60.03	1 : 2.6

remained unchanged in both fractions after incubation with phospholipase A_2. This probably depends upon the fact that about 40% of mitochondrial proteins was lost after 30 min incubation with phospholipase A_2. In outer membranes, 5 min incubation with phospholipase induced a 25% loss of protein content. The cholesterol to phospholipid molar ratio underwent great increases in both lipid-deficient mitochondria and lipid-deficient outer membranes. When phospholipid vesicles were added, the phospholipid content increased 5 times in whole mitochondria and 8 times in outer membranes, while the cholesterol content did not change significantly. This caused the cholesterol to phospholipid molar ratio to decrease to 1 : 12.0 in whole mitochondria and 1 : 9.6 in isolated outer membranes. Finally, the lipid-deficient fractions, incubated with cholesterol-containing phospholipid vesicles, exhibited a sharp rise of both cholesterol content and cholesterol to phospholipid molar ratio.

Discussion

Different enzymatic activities of mitochondria have been associated with the outer membrane [29]. However, among these activities a lipid-dependence has been only demonstrated for kynurenine hydroxylase [8]. The results in this paper propose an activatory role of phospholipids for another enzyme linked to the outer mitochondrial membrane [29], NADH-cytochrome *c* reductase (rotenone-insensitive). The lipid depletion of mitochondria, either induced by acetone extraction or digestion with phospholipase A_2 or phospholipase C, resulted in the inhibition of the reductase activity. The inactive enzyme was reactivated by phospholipids. Both the enzyme inactivation as well as the enzyme reactivation were shown to correlate well with the amounts of lipid removed from mitochondria and the amounts of lipid added to lipid-deficient mitochondria, respectively. The experiments with isolated outer membranes essentially confirmed these results, indicating that the effects of the lipid depletion and lipid rebinding, observed in whole mitochondria, were specific to the outer membrane.

A mixture of mitochondrial phospholipids was found to be more efficient for high enzyme reactivation than the individual phospholipid tested. With acetone-treated and phospholipase A_2-digested mitochondria, full reactivation occurred in the presence of 2.3 and 3.6 μmol phospholipid/mg protein, respectively. With isolated outer membranes submitted to phospholipase A_2, maximal reactivation was attained with 4.8 μmol phospholipid/mg protein. These figures agree with those described by various authors for full reactivation of mitochondrial enzymes by phospholipid mixtures or individual phospholipids [8,13,30—32]. Among the individual phospholipid tested, phosphatidylcholine was shown to be the most active for the restoration of NADH-cytochrome *c* reductase (rotenone-insensitive) activity in both lipid-deficient mitochondria and lipid-deficient outer membranes. However, the maximal restoration with this phospholipid was not higher than 53%. Thus, it appears that none of the phospholipids existing in the outer mitochondrial membrane (cf. ref. 27) were specifically required for interaction with the active protein. Perhaps this phenomenon may be explained on the basis of the observation that in mixtures of different phospholipids, complex physicochemical changes

occurs in the lipid molecules [33]. The mesomorphic transitions of biionic phospholipids are strongly influenced by the incorporation of small amounts of anionic lipids. In this way mixtures of different phospholipids could exhibit a degree of fluidity which differs from that of the individual components. This fluidity could represent the physical condition required for stabilization of the active protein (cf. ref. 34) and its optimal functioning. The physicochemical changes in phospholipid mixtures must be considered speculative in this study, but they provide a possible explanation for the results.

An alternative hypothesis to explain the enzyme activation by phospholipids could be that the lipid addition to lipid-deficient preparations results in the release of enzyme inhibition induced by acetone or free fatty acids produced during incubation with phospholipase. However, acetone inhibition is excluded in experiments with phospholipases. In addition, it has been shown that repeated washings with mixtures containing high albumin concentrations are able to completely remove fatty acids as well as lysophosphatides from phospholipase-digested mitochondria [13]. The possibility that trace amounts of fatty acids remain in membranes after washing and cause enzyme inhibition seems to be discounted by the observation that relatively high concentrations of various detergents were not able to fully activate the reductase in lipid-deficient mitochondria. The addition of detergents to lipid-deficient membranes should determine high detergent/lipid ratio, which represent a favourable condition for fatty acids solubilization [35]. The small enzyme activation by detergents could rather indicate that, at least in the adopted conditions, detergents were not able to create the required physicochemical conditions for full restoration of the reductase activity.

A preferential grouping of some lipid molecules on the basis of differential binding to proteins of different fluidity seems to occur near the enzyme molecules in membranes [19,20,36]. Reconstitution experiments have stressed the importance of the length and unsaturation of the phospholipid paraffin chains of such a lipid microenvironment for the activity of several enzymes [37—39]. The inhibition of the native as well as reconstituted reductase at low temperatures, could be a consequence of the temperature-induced transition to the gel state of the lipid near the enzyme molecules. This interpretation is supported by the inhibition of the reductase stimulation when dipalmitoyl phosphatidylcholine was added to lipid-deficient mitochondria in place of egg phosphatidylcholine. The latter phospholipid contains both unsaturated and saturated fatty acids, which at the temperature of the enzyme assay (30°C) are in a fluid state [40]. Dipalmitoyl phosphatidylcholine exhibits transition from the gel to the smectic liquid-crystalline form at 41—45°C [40]. The "freezing" of the hydrocarbon phase at 30°C could decrease the motion of the enzyme molecules or limit their penetration into the bilayer.

An analogous explanation may be proposed for cholesterol's effect on NADH-cytochrome *c* reductase (rotenone-insensitive). Cholesterol added to lipid-deficient mitochondria or lipid-deficient outer membranes together with mitochondrial phospholipids prevents the stimulation of the reductase activity. A similar effect of cholesterol was described for $(Na^+ + K^+)$-ATPase [41] and β-hydroxybutyrate dehydrogenase [39]. The onset temperature of endothermal transition from gel to the liquid-crystalline form for the lipids of

the outer mitochondrial membrane was found to be −15°C [42]. The incorporation of cholesterol between phospholipid molecules above the transition temperature induces lipids to become less fluid [28], thus restricting the molecular motion in the functional areas of membranes. Our results indicate that a cholesterol to phospholipid molar ratio of 1 : 9.6 in reconstituted outer membranes is compatible with high reductase activity. When the ratio rises to 1 : 2.6 a greater number of phospholipid molecules will form complexes with cholesterol. As a consequence, fluid functional areas in membranes should undergo a dramatic decrease, which then results in enzyme inhibition.

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