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Reassociation of Soluble Monoamine Oxidase with Lipid-Depleted Mitochondria in the Presence of Phospholipids*

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ABSTRACT: It has previously been shown that the monoamine oxidase of pig liver mitochondria can be rendered buffer soluble by extraction of the mitochondria with ethyl methyl ketone in the presence of ammonium sulfate and that the crucial point in the extraction procedure is to remove the highly acidic phospholipids from the mitochondria. By sucrose density gradient centrifugation and by gel filtration it was found that the enzyme forms soluble complexes with some highly acidic phospholipids but not with phosphatidylcholine.

When enzyme solution was mixed with a phospholipid dispersion and lipid-depleted mitochondria were then added, the enzyme bound to the mitochondrial residues. Such binding was observed with all highly acidic phospholipids tested, whereas phosphatidylcholine and phosphatidylethanolamine caused no binding. The effect on the binding of various amounts of cardiolipin, enzyme and lipid-depleted mitochondria was studied, but no simple stoichiometry was found. The lowest ratio of cardiolipin to rebound enzyme observed was about eight by weight, indicating that relatively large amounts of phospholipid were necessary for the binding. Binding occurred also with lipid-depleted red cell membranes and with lipid-depleted milk fat globule membranes. The prior formation of soluble enzyme–phospholipid complexes was not necessary for the binding since binding also occurred when lipid-depleted mitochondria were first relipidated and then added to enzyme solution. The extent of binding was higher at 37° than at 4° and was slightly decreased by high ionic strength. The rebound enzyme could not be extracted from the mitochondrial residues by buffer. It is concluded that this monoamine oxidase can interact with highly acidic phospholipids either in solution, forming soluble complexes, or with the same phospholipids bound to insoluble membrane residues, then forming insoluble complexes.

The major constituents of biological membranes are proteins and polar lipids. How these constituents are arranged to form the membrane is not known, although several detailed models have been proposed. One approach to this problem is the study of interactions between membrane proteins and polar lipids. Hollunger and Oreland (1970) have recently described a method for obtaining monoamine oxidase from pig liver mitochondria in water-soluble form by a two-step extraction with ethyl methyl ketone. L. Oreland and T. Olivecrona (to be published) have subsequently shown that extraction of the acidic phospholipids from the mitochondria is the crucial point in the extraction procedure and have proposed that the enzyme is bound to the mitochondrial structure *in vivo* by interaction with such phospholipids. In the present paper we show that the soluble enzyme rebinds to lipid-depleted mitochondria when anionic phospholipids are added. Since the enzyme activity can be measured by a simple spectrophotometric assay and is the same in the presence or absence of added phospholipids, we consider this a useful system in which to study the interaction between a membrane enzyme and phospholipids.

Materials

Phosphatidylcholine and Phosphatidylethanolamine. Two egg yolks were homogenized in chloroform–methanol (2:1, v/v) and the final volume of solvent adjusted to 250 ml. The homogenate was filtered and 50 ml of aqueous 0.01 M $CaCl_2$ was added. The mixture was allowed to separate into two layers. To remove nonlipid contaminants, the lower, chloroform, solution of lipid was passed through a column of cellulose powder and eluted with a further 50 ml of chloroform. The combined eluates were concentrated to 10 ml and poured into 100 ml of acetone (4°). The precipitate

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TABLE I: Fatty Acid Composition of Phospholipid Preparations.[a]

	16:0	16:1	18:0	18:1	18:2	20:1[b]	20:2	22:6[b]
Egg phosphatidylcholine	32.0	1.0	16.0	33.3	12.7		2.7	2.3
Egg phosphatidylethanolamine	17.6		27.4	25.0	8.7		12.9	8.3
Brain phosphatidylserine			51.2	41.6		7.2		
Pea phosphatidylinositol	46.9		5.4	12.1	30.0	5.6		
Phosphatidic acid	35.9	2.0	13.6	32.6	13.5		2.4	
Ox heart cardiolipin[c]	4.4	4.4	3.0	9.0	73.4	5.8		

[a] Lipid extracts of samples of the sonicated phospholipid dispersions were taken to dryness and methyl esters of the fatty acids prepared by transmethylation. Gas chromatography was performed on a Hewlett-Packard Model 5750 using diethylene glycol succinate as stationary phase. Peaks were identified by comparison with authentic standards. [b] Tenetative identification. [c] Purified by thin-layer chromatography.

was dissolved in ether (20 ml) and again poured into cold acetone (4°). The precipitate was collected and dissolved in chloroform–methanol (4:1, v/v). It was then chromatographed on a silicic acid column (Bio-Sil, Bio-Rad Laboratories, Richmond, Calif.) in the same solvent. Fractions were collected as elution proceeded and samples from them were analyzed for phosphorus. Two main peaks were obtained, the first containing phosphatidylethanolamine, the second phosphatidylcholine. These substances were further purified by silicic acid thin-layer chromatography with chloroform–methanol–acetic acid–water (25:15:4:2, v/v) as developing solvent. Zones were visualized by spraying a strip of the chromatogram with a solution of iodine in methanol. The zones migrating as phosphatidylcholine and phosphatidylethanolamine, respectively, were scraped off, transferred to small glass columns, and eluted with chloroform–methanol–acetic acid–water (50:39:1:10, v/v). Three-tenths volume of 4 M aqueous ammonia was added. The phases were allowed to separate and the lower, chloroform, phase was evaporated to dryness.

Phosphatidylserine. Crude phosphatidylserine was prepared from ox brain as described by Ansell and Hawthorne (1964). This material was further purified by thin-layer chromatography with chloroform–methanol–25% aqueous ammonia (65:35:5, v/v). On these plates a distinct separation of phosphatidylserine from phosphatidylinositol was obtained. The zone corresponding to phosphatidylserine was localized and eluted as described for phosphatidylcholine. Three-tenths volume of 0.5 M NaCl was added to the eluate. The phases were allowed to separate and the lower, chloroform, phase was evaporated to dryness.

Phosphatidylinositol. A crude phosphatidylinositol was prepared from frozen peas as described by Ansell and Hawthorne (1964). This material was further purified on silicic acid thin-layer chromatography in the same system as used for phosphatidylserine.

Radioactively Labeled Phosphatidylinositol Plus Phosphatidylserine. ^{14}C-Labeled linoleic acid (The Radiochemical Centre, Amersham, England) in 5% bovine serum albumin was injected intravenously to an ether-anesthetized rat. Five minutes later the rat was killed. Liver lipids were extracted by chloroform–methanol (2:1, v/v). Phosphatidylserine plus phosphatidylinositol was isolated by thin-layer chromatography on silica gel G with chloroform–methanol–acetic acid–water (25:15:4:2, v/v) as developing solvent. This fraction was eluted as described for phosphatidylserine.

Phosphatidic Acid. This material was prepared by enzymatic hydrolysis of purified egg yolk phosphatidylcholine essentially as described by Papahadjopoulos and Miller (1967). After chromatography on a silicic acid column as described by these authors, the material was further purified by silicic acid thin-layer chromatography using a two-step elution. The first solvent was acetone–petroleum ether (bp 30–60°) (1:3, v/v), followed by chloroform–methanol–acetic acid–water (80:13:8:0.3, v/v). The zone migrating as phosphatidic acid was scraped off, transferred to a small glass column, and eluted with ethanol–chloroform–water (10:3:2, v/v).

Cardiolipin. Ox heart ventricles were homogenized in chloroform–methanol (2:1, v/v), 20 volumes/g of tissue. The homogenate was filtered into a separatory funnel, 8 volumes of aqueous 2% KH_2PO_4 was added, and the funnel was shaken. After separation of the phases, the lower, chloroform, phase was recovered, dried over anhydrous sodium sulfate, concentrated, and applied to a silicic acid column. The column was first eluted with chloroform to remove neutral lipids, 5% methanol in chloroform then eluted a "crude cardiolipin" fraction. In some experiments (Tables I and II), this material was further purified on silicic acid thin-layer chromatography with chloroform–methanol–water–aqueous ammonia (108:50:5:2.85, v/v) as developing solvent. The zone migrating as cardiolipin was eluted as described for phosphatidic acid. In other experiments the "crude cardiolipin" was purified by precipitation as the barium salt as described by Ames (1968) and was converted into the sodium salt by passage through a column packed with Dowex 50 (Na^+) form as described by the same author. In yet other experiments cardiolipin obtained from Koch-Light Laboratories (Colnbrook, England) or from Pierce Chemical Co. (Rockland, Ill.) was used.

Preparation of Phospholipid Dispersions. The phospholipid preparations were taken to dryness and 1 ml of 0.001 M EDTA in 0.01 M potassium phosphate buffer (pH 7.2) per 10 mg of phospholipid was immediately added. The mixture was then sonicated for 10–15 min at 25–35° in a thermostated, jacketed vessel using the MSE 100-W ultrasonic disintegrator. The resulting dispersions were dialyzed against several changes of the same buffer for at least 24 hr. They were then centrifuged at 43,000*g* for 30 min in a Sorvall RC 2 B centrifuge. Any sediment was discarded. A sample of the supernatant was extracted with chloroform–methanol (2:1, v/v). Nonlipids were washed away by addition of four-tenths volume of 0.15 M NaCl and the lipid content of the chloroform phase estimated by determination of lipid phosphorus. Another sample was used for preparation of methyl esters of the fatty

TABLE II: Effect of Purified Phospholipids on the Binding of Soluble Monoamine Oxidase to Lipid-Depleted Mitochondria.

Phospholipid Added[a]	Monoamine Oxidase Remaining in the Supernatant: % of Control[b]		Lipid P Remaining in the Supernatant: % of the Amt Added[b]	
Egg phosphatidylcholine	104	102	97	97
Egg phosphatidylethanolamine	100	95	13	7
Brain phosphatidylserine	36	34	*c*	3
Pea phosphatidylinositol	36	38	7	4
Phosphatidic acid	25	28	3	4
Cardiolipin	27	24	*c*	*c*

[a] The phospholipid dispersions were centrifuged at 43,000*g* for 30 min before use. In each experiment about 6 μmoles of phospholipid was added. [b] About 600 units of soluble monoamine oxidase was incubated for 5 min with the dispersed phospholipid in a total volume of 0.7 ml of 0.01 M potassium phosphate buffer (pH 7.2) containing 0.001 M EDTA and then added to 3 mg of lipid-depleted mitochondrial residues. After mixing with a glass rod the suspension was incubated for 5 min and then centrifuged at 43,000*g* for 10 min. The supernatant was analyzed for monoamine oxidase activity and lipid P (see Methods). The same experiment without the addition of phospholipid was used as control. All procedures were performed at 0–4°. [c] Not measurable.

acids for gas chromatography. Results of this analysis are shown in Table I.

Methods

Preparations of Soluble Monoamine Oxidase and of Lipid-Depleted Mitochondria. Soluble monoamine oxidase was prepared from pig liver mitochondria that had been washed with water (Hollunger and Oreland, 1970). In a first extraction step the mitochondrial suspension was extracted with 8 volumes of ethyl methyl ketone and the residue was then washed with buffer. In a second extraction step the residue from the first extraction was suspended in 0.05 M ammonium sulfate and extracted with 11 volumes of ethyl methyl ketone. From the resulting residue about 25% of the original enzyme activity was extracted into 0.1 M phosphate buffer (pH 7.2) containing 0.001 M EDTA. Before use the buffer extract was dialyzed against several changes of 0.01 M potassium phosphate buffer (pH 7.2) containing 0.001 M EDTA.

The residue obtained after the extraction procedure contained phospholipids and protein in a ratio about 1 to 30 that in the mitochondrial preparation (Hollunger and Oreland, 1970), and was designated lipid-depleted mitochondria. Before use it was washed once with distilled water to remove any soluble protein.

Assay for Monoamine Oxidase Activity. In all experiments monoamine oxidase activity was measured by the spectrophotometric method of Tabor *et al.* (1954) using benzylamine as substrate. One unit of enzyme activity was defined as the amount of enzyme causing a change in absorbance at 250 nm of 0.001/min in a cuvet with 1-cm light path containing 10 μmoles of benzylamine (neutralized) and 0.01 M potassium phosphate buffer (pH 7.2) to 3 ml at 24°.

Protein Estimation. Protein was estimated by the method of Lowry *et al.* (1951) with human serum albumin as standard.

Determination of Lipid Phosphorus. The phospholipids were extracted by the method of Folch *et al.* (1951). The dried extracts were digested over a microburner with 0.1 ml of a 2:1 (v/v) mixture of concentrated H_2SO_4 and 70% $HClO_4$. Inorganic phosphate was then determined by the method of Chen *et al.* (1956).

Results

When pig liver mitochondria are extracted with ethyl methyl ketone by the two-step procedure described by Hollunger and Oreland (1970) (see Methods) about one-fourth of the monoamine oxidase activity can be extracted by buffer. This enzyme does not sediment when centrifuged for 1 hr at 100,000*g*. The present experiments concern the binding back of soluble enzyme to the lipid-depleted mitochondria by phospholipids. In most of the experiments a buffer solution of the enzyme and a phospholipid dispersion were added to lipid-depleted mitochondria. After a brief incubation, the mixture was centrifuged 10 min at 43,000*g* and the amount of enzyme left in solution was measured. If the lipid-depleted mitochondria are incubated in buffer only, a small amount of enzyme is extracted from them. If they are incubated in a buffer solution of soluble enzyme, we often find slightly more enzyme in solution after the incubation than was added, which means that additional enzyme has been brought into solution. In our studies with phospholipids we have therefore always used control incubations with lipid-depleted mitochondria and a buffer solution of the enzyme and have expressed the enzyme activity in the test incubation as per cent of that in the control incubation.

Effect of Dispersions of Purified Phospholipids (Table II). Phosphatidylserine, phosphatidylinositol, phosphatidic acid, and cardiolipin all caused most of the soluble enzyme to rebind to the lipid-depleted mitochondria. In contrast, phosphatidylcholine and phosphatidylethanolamine had no measurable effect. We also tried dispersions of mixed phospholipids but found no effect with soy bean phospholipids or with crude egg lecithin. It is possible that the difference in effect among different phospholipid dispersions are related to their charges, since all the effective phospholipids are negatively charged at pH 7.2, whereas phosphatidylcholine has no net charge at this pH and phosphatidylethanolamine has only a small net negative charge. The mixed phospholipid preparations mentioned contain mainly phosphatidylcholine and phosphatidylethanolamine and thus have only small net negative charges. All the effective phospholipids bound efficiently to the lipid-depleted mitochondria, whereas there was no significant binding of phosphatidylcholine. However, the correlation between the binding of the phospholipid dispersions to the lipid-depleted mitochondria and their capacity to bind monoamine oxidase to the mitochondria was not complete since phosphatidylethanolamine bound to the lipid-depleted mitochondria but had no effect on the soluble enzyme.

When the phospholipid dispersions were mixed with a buffer solution of the enzyme, the enzyme activity in solution was the same as in corresponding controls without phospholipids both before and after centrifugation. Thus, the phospholipid dispersions did not cause inactivation of the soluble enzyme

TABLE III: Binding of Soluble Monoamine Oxidase to Some Ion Exchangers.[a]

Ion Exchanger	Monoamine Oxidase in the Supernatant
DEAE-Sephadex	100
CM-Sephadex	3500
SE-Sephadex	3200
Sephadex G-25[b]	3850

[a] 0.1 g of DEAE-Sephadex A-50, CM-Sephadex G-50, and SE-Sephadex C-50, respectively, were equilibrated twice with 30 ml of 0.1 M potassium phosphate buffer (pH 7.2) and twice with 30 ml of 0.01 M potassium phosphate buffer (pH 7.2). Soluble monoamine oxidase in 0.01 M potassium phosphate buffer (pH 7.2) was then added to each ion exchanger and the total volume was adjusted to 10 ml with the buffer. The mixtures were stirred and then centrifuged at 5000*g* for 5 min. The supernatants were sucked off and their enzyme activities determined. All procedures were carried out at 0–4°. [b] As a control the same procedure was performed with 0.1 g of Sephadex G-25.

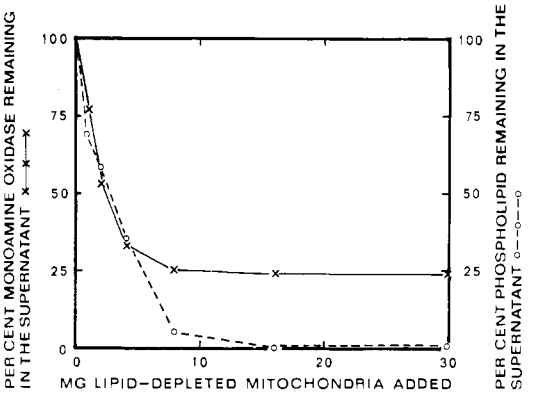

FIGURE 1: The effect of various amounts of lipid-depleted mitochondria on the binding of cardiolipin and soluble monoamine oxidase. Dispersed cardiolipin (1.0 μmole) and a soluble monoamine oxidase preparation (350 units) in 0.5 ml of 0.01 M potassium phosphate buffer (pH 7.2), containing 0.001 M EDTA, was added to the amount of lipid-depleted mitochondria indicated. The experiments were then carried out as described in the legend to Table II. Corrections were made for the amount of enzyme obtained in the supernatant after incubation of the various amounts of lipid-depleted mitochondria without the addition of soluble enzyme.

or bring soluble enzyme out of solution in the absence of lipid-depleted mitochondria.

It is possible that the phospholipids bind to the lipid-depleted mitochondria in such a way that a large proportion of their negatively charged heads are directed toward the aqueous environment. It seemed possible that the enzyme might bind electrostatically to such a structure. To test this possibility we studied the binding of the enzyme to some ion exchangers (Table III). The enzyme was bound by the anion exchanger DEAE-Sephadex but not by the cation exchangers SE-Sephadex or CM-Sephadex. The enzyme could be eluted from DEAE-Sephadex by 0.5 M NaCl in 0.01 M potassium phosphate buffer (pH 7.2), thus demonstrating that it was bound but not inactivated by this ion exchanger. The binding is in good agreement with the estimated isoelectric point for the soluble enzyme of 4.7 (L. Oreland, unpublished data). At pH 7.2 the enzyme has a net negative charge and binds to a positively charged matrix, but not to a negatively charged matrix.

Effects of Variations in the Proportions of Phospholipids, Lipid-Depleted Mitochondria, and Enzyme. These experiments were carried out with cardiolipin preparations (see Methods). Cardiolipin was chosen since it is one of the major phospholipids of mitochondria (Rouser *et al.*, 1968) and is effective in our system.

When the amount of lipid-depleted mitochondria was varied but the amounts of phospholipids and of monoamine oxidase were kept constant (Figure 1), the amount of phospholipid bound increased with increasing amounts of added lipid-depleted mitochondria. When 8 mg of lipid-depleted mitochondria had been added, essentially all the phospholipid was bound, corresponding to a phospholipid to protein ratio of about 0.18 by weight. This ratio is about 0.16 in pig liver mitochondria washed twice with water (Hollunger and Oreland, 1970). The binding of enzyme paralleled the binding of phospholipid when 4 mg or less of lipid-depleted mitochondria was added. With 8, 16, and 30 mg of lipid-depleted mitochondria about three-fourths of the added enzyme was bound.

When the amount of cardiolipin was varied, but the amount of lipid-depleted mitochondria was kept constant (Figure 2) the amount of enzyme bound increased with increasing amounts of added phospholipid and approached a plateau value. The proportion of phospholipid to lipid-depleted mitochondria never exceeded 0.13 by weight. Thus, there was always enough lipid-depleted mitochondria present to bind essentially all the added phospholipid. Three different amounts of soluble enzyme were used, covering a tenfold range of enzyme concentration. When the amount of added

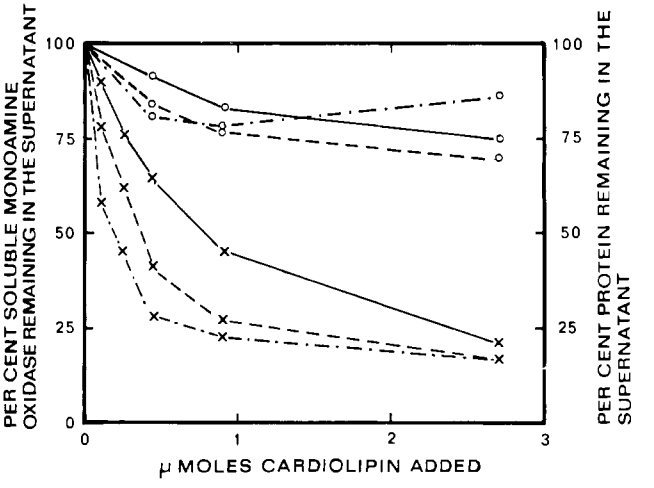

FIGURE 2: The effect of various amounts of cardiolipin on the binding of soluble monoamine oxidase and other proteins to lipid-depleted mitochondria; 0.05, 0.2, and 0.5 ml of the buffer extract (containing soluble monoamine oxidase 3000 units/ml and total protein 3.2 mg/ml) and the indicated amounts of dispersed cardiolipin, all in 0.01 M potassium phosphate buffer (pH 7.2), containing 0.001 M EDTA to a final volume of 0.7 ml, were incubated with 30 mg of lipid-depleted mitochondria as described in the legend to Table II. Monoamine oxidase activity and protein were estimated as described in Methods. Buffer extract (0.5 ml): x—x—x, monoamine oxidase activity; o—o—o, protein; buffer extract (0.2 ml): x--x--x, monoamine oxidase activity; o--o--o, protein; buffer extract (0.05 ml): x···x···x, monoamine oxidase activity, o···o···o, protein.

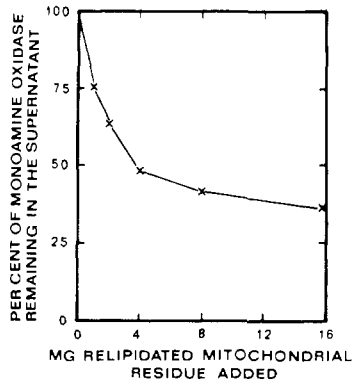

FIGURE 3: The effect of various amounts of relipidated mitochondria on the binding of soluble monoamine oxidase. Various amounts of lipid-depleted mitochondria were incubated with dispersed cardiolipin for 5 min in 0.01 M potassium phosphate buffer (pH 7.2), containing 0.001 M EDTA. In all cases cardiolipin was added in excess to saturate the lipid-depleted mitochondria. After centrifugation at 43,000*g* for 10 min the supernatant was removed. Soluble monoamine oxidase in 0.7 ml of the phosphate buffer was then added to the relipidated mitochondria and the suspensions incubated for 5 min. The suspensions were then centrifuged at 43,000*g* for 10 min and the supernatants analyzed for monoamine oxidase activity as described in Methods. The same experiment without the addition of cardiolipin was used as control. All procedures were performed at 0–4°.

phospholipid was high, the percentages of enzyme bound at all three enzyme concentrations were similar. At low amounts of added phospholipid, however, the percentages of enzyme that were bound differed. If the same absolute amounts of enzyme were bound, the ratio of percentages bound in our experiments should be 1:4:10. At the lowest amount of added phospholipid, the ratio was 1:2.2:4.2. It is possible that the theoretical ratio could be reached at lower amounts of added phospholipid but we have not been able to test this, since the amount of enzyme that becomes bound would then be too low to be reliably measured.

The binding was relatively specific for monoamine oxidase. This enzyme represents about 10% of the added protein (L. Oreland, unpublished data), but whereas at high amounts of added cardiolipin three-fourths of the added enzyme was bound, only about one-fourth of the added protein was bound (Figure 2). Thus all proteins in the buffer solution were not equally bound to the lipid-depleted mitochondria in the presence of cardiolipin, although these proteins must have many properties in common since they were extracted in the same, rather specific, way.

The amount of enzyme bound was not directly proportional to the amount of phospholipid added. Thus, no simple stoichiometry can be calculated. However, the lowest ratio of added phospholipid to re-bound enzyme in the experiment in Figure 2 was about eight by weight. This ratio was obtained at the lowest amount of added phospholipid and the highest enzyme concentration. The molecular weight of the enzyme has been estimated as 115,000 (L. Oreland, unpublished data). From these data it can be calculated that the molar ratio of added phospholipid to re-bound enzyme was at least 600 in the experiments in Figure 2. Thus, the amounts of phospholipid required to cause binding of the enzyme were relatively large, much larger then expected if the only effect of the added phospholipid was to saturate a distinct number of binding sites on the enzyme to give a complex which then bound to the lipid-depleted mitochondria.

TABLE IV: Ability of Ethyl Methyl Ketone Extracted Membranes from Various Sources to Bind Cardiolipin and Soluble Monoamine Oxidase.[a]

Source of Membranes	Monoamine Oxidase in the Supernatant: % of Control	Lipid P in the Supernatant: % of Amt Added
Pig liver mitochondria	21	*d*
Red cells[b]	15	*d*
Milk fat globules[c]	24	*d*
Bakers' yeast	95	89

[a] About 600 units of soluble monoamine oxidase was incubated for 5 min with 1.4 μmoles of dispersed cardiolipin in a total volume of 0.7 ml of 0.01 M potassium phosphate buffer (pH 7.2) containing 0.001 M EDTA, and then added to lipid-depleted residues from 80 mg of membranes extracted by the ethyl methyl ketone procedure described in Methods. The experiments were carried out as described in the legend to Table II. [b] Red cell membranes were isolated as described by Rega *et al.* (1967). [c] Cream (50% fat) was obtained by centrifugation of unpasteurized bovine milk. The cream was washed twice with an equal volume of water by centrifugation and then churned. The buttermilk was lyophilized before extraction with ethyl methyl ketone. [d] Not measurable.

Binding of Phospholipid to Lipid-Depleted Mitochondria Prior to Incubation with Enzyme. Two different sequences of events might take place in the three-component system. The enzyme and the phospholipids in solution might form a lipoprotein complex which then binds to the mitochondrial structure. Alternatively, the phospholipids might first bind to the lipid-depleted mitochondria and the enzyme then bind to this lipoprotein matrix. These two possibilities are not mutually exclusive. Figure 3 show the results of an experiment designed to test these possibilities. Lipid-depleted mitochondria were mixed with a dispersion of cardiolipin, centrifuged, and the supernatant removed. A buffer solution of enzyme was then added. Some of the added enzyme became bound to the relipidated mitochondrial residues. When more relipidated mitochondria were added, more enzyme became bound, but there was no direct proportionality. This curve resembles that in Figure 2, *i.e.*, the results were similar whether the phospholipids were bound to the mitochondrial residues before the addition of enzyme or whether the enzyme was preincubated with phospholipids before being added to the lipid-depleted mitochondria. However, in several experiments we have consistently found that the same amounts of mitochondrial residues and phospholipids are less efficient in binding the enzyme when the phospholipids are first bound to the mitochondrial residues than when enzyme and phospholipids are mixed prior to the addition of lipid-depleted mitochondria.

The reason for the nonlinearity of the binding of soluble enzyme to the relipidated mitochondria was studied in a further experiment. Enzyme solution was added to relipidated mitochondria in approximately the same proportions as for the point to the far right in Figure 3; 40% of the added

TABLE V: Effect of Temperature on the Binding of Soluble Monoamine Oxidase to Lipid-Depleted Mitochondria by Phospholipids.[a]

Phospholipid Added	Temp (°C)	Monoamine Oxidase Remaining in the Supernatant: % of Control
Phosphatidylcholine	0	105
	37	98
Phosphatidylethanolamine	0	100
	37	96
Cardiolipin	0	30
	37	17

[a] The experiments were performed as described in the legend to Table II. Constant temperature was maintained by the use of a thermostated water bath or an ice bath.

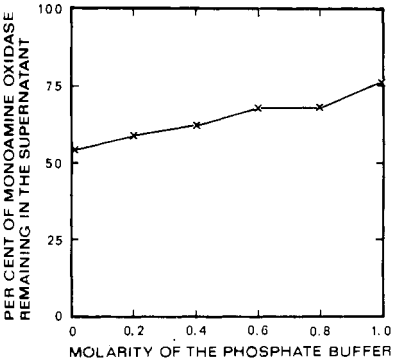

FIGURE 4: The effect of salt concentration of the binding of soluble monoamine oxidase to relipidated mitochondrial residues. To 30 mg of lipid-depleted mitochondria was added 1.7 μmoles of dispersed cardiolipin in 0.01 M potassium phosphate buffer (pH 7.2), containing 0.001 M EDTA. The suspensions were incubated for 5 min, centrifuged for 10 min at 43,000*g*, and the supernatants removed. To the relipidated residues was added soluble monoamine oxidase in 0.7 ml of potassium phosphate buffer of various molarities and the suspensions incubated for 5 min. After centrifugation at 43,000*g* for 10 min, monoamine oxidase activity was estimated in the supernatants (see Methods). The same experiment without the addition of cardiolipin was used as control. All procedures were performed at 0–4°.

enzyme remained in the supernatant after centrifugation. A new, equal, portion of enzyme solution was added to the sediment, and the mixture was stirred and centrifuged. Again about 40% of the added enzyme remained in the supernatant. Thus, during the first incubation the relipidated mitochondria had not exhausted their capacity to bind the enzyme. When fresh, relipidated mitochondria were added to the supernatant, no further binding of enzyme occurred. Thus, it appears that in this experiment the percentage of enzyme bound depended on a property of the enzyme and not on the relipidated mitochondria. When the supernatant was mixed with a dispersion of cardiolipin and then added to lipid-depleted mitochondria, only 30% of the enzyme (12% of the enzyme activity of the original enzyme solution) remained in solution after centrifugation. Thus, some of the enzyme interacted with cardiolipin so that it then bound to the lipid-depleted mitochondria.

Effect of Other Insoluble Lipid-Depleted Membrane Preparations. Monoamine oxidase is probably present only in mitochondria (Schnaitman *et al.*, 1967). To test whether the observed binding is specific for mitochondrial membranes we extracted some other membrane preparations in a manner analogous to our preparation of lipid-depleted mitochondria. Red cell membranes and milk fat globule membranes were chosen since they contain no mitochondria. Yeast was chosen as a source of membranes from a very different organism. The red cell membrane and the fat globule membrane preparations both bound cardiolipin and also soluble monoamine oxidase to a similar extent as did the lipid-depleted mitochondria, whereas the yeast preparation bound neither cardiolipin nor enzyme (Table IV). None of the membrane preparations bound enzyme in the absence of added phospholipid. This experiment shows that the binding of monoamine oxidase in the presence of cardiolipin does not specifically require mitochondrial membrane residues, but that some other membrane residues which can bind the phospholipid also can bind the enzyme.

Effect of Temperature. Other investigators (Lenaz *et al.*, 1969) have shown that the binding of phospholipids to lipid-depleted mitochondria is increased by increased temperature. In our system (Table V), the binding of enzyme to the lipid-depleted mitochondria in the presence of cardiolipin was more efficient at 37° than at 4°. With phosphatidylcholine and with phosphatidylethanolamine, however, no binding was observed at either temperature.

Effect of Salts. The binding was less efficient at high salt concentrations (Figure 4). Cardiolipin was first bound to lipid-depleted mitochondria at a low ionic strength and the relipidated mitochondrial residues were then incubated with enzyme solution at different potassium phosphate concentrations. Thus, the effect of salt probably was not on the binding of phospholipid to the lipid-depleted mitochondria, although it is possible that the salt caused some rearrangement in the lipoprotein structure. The effect of salt was less than expected if the binding occurred through electrostatic interaction alone (Jacobs and Sanadi, 1960). When calcium ions are added to a dispersion of anionic phospholipids the charge at their plane of shear is changed. If this charge is important in our system, calcium ions should influence the binding of enzyme in the presence of cardiolipin. However, when enzyme solution was added to relipidated mitochondria, the presence or absence of 0.01 M $CaCl_2$ caused no difference in the binding of enzyme.

Nonreversibility of the Binding. In other experiments we found that when the enzyme had become bound to relipidated mitochondrial residues, it could not be extracted by a buffer, irrespective of the ionic strength of the buffer (0.001–1 M phosphate buffer, pH 7.2, was tried). Therefore, the results of the previous experiments cannot be considered in terms of a distribution between bound and nonbound enzyme in a reversible system. Similarly, the enzyme in intact mitochondria cannot be extracted by buffer without the addition of a detergent (Gorkin, 1966). In contrast, the extraction of enzyme from mitochondria delipidated by the procedure of Hollunger and Oreland (1970) is more efficient with buffers of high ionic strength than with buffers of low ionic strength (L. Oreland, unpublished data). Preliminary experiments indicate that monoamine oxidase rebound to mitochondria by cardiolipin, can once again be liberated by the ethyl

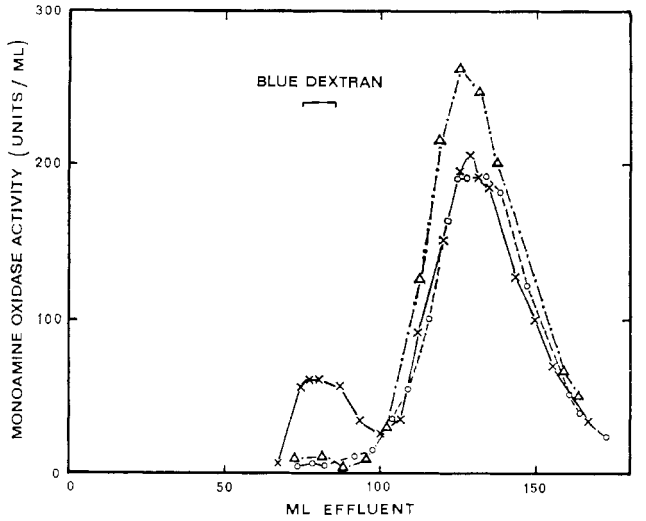

FIGURE 5: Gel filtration on Sepharose 4B of soluble monoamine oxidase in the presence of phospholipid dispersions. Buffer extract containing soluble monoamine oxidase (10,000 units), o---o---o---o; an identical preparation + 7 μmoles of dispersed phosphatidylcholine, Δ-·-·-Δ-·-·-Δ; and an identical enzyme preparation + 7 μmoles of dispersed cardiolipin, x—x—x; all in 2 ml of 0.01 M potassium phosphate buffer (pH 7.2), containing 0.001 M EDTA, were applied to a 2.5 × 40 cm column of Sepharose 4B (Pharmacia, Sweden), equilibrated with the same buffer. Elution was carried out with the phosphate buffer, and monoamine oxidase activity estimated on the fractions (see Methods).

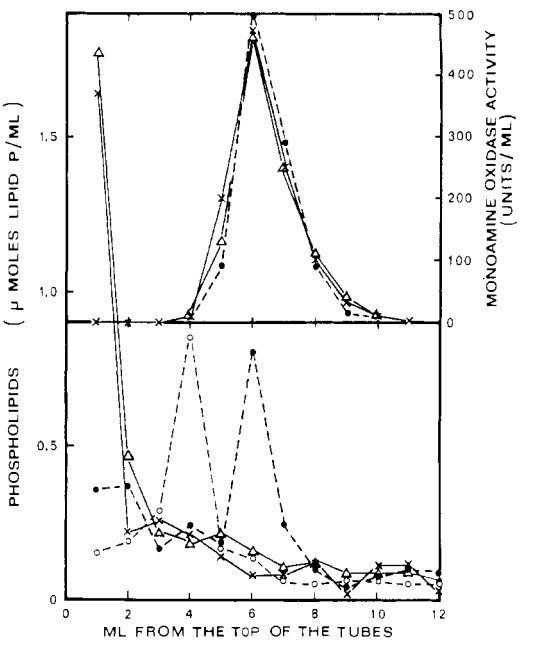

FIGURE 6: Sucrose density gradient centrifugation of soluble monoamine oxidase in the presence of dispersed phosphatidylcholine and cardiolipin. Sucrose density gradients were prepared in five tubes by layering 1 ml each of 40, 35, 30, 27, 24, 20, 17, 14, and 3 ml of 10% sucrose in 0.01 M potassium phosphate buffer (pH 7.2), and 0.001 M EDTA. On the sucrose gradients were layered in 1 ml of the phosphate buffer, respectively: soluble monoamine oxidase preparation (1100 enzyme units); 4.0 μmoles of dispersed phosphatidylcholine; 1.4 μmoles of cardiolipin; 4.0 μmoles of dispersed phosphatidylcholine + 1100 units of monoamine oxidase; and 1.4 μmoles of cardiolipin + 1100 units of monoamine oxidase. The tubes were centrifuged at 175,000*g* for 16 hr at 4° in a Spinco rotor SW-40. The contents of the tubes were then fractionated by suction through a U-shaped needle from the top. Monoamine oxidase activity and lipid P were determined as described in Methods. The upper part of the figure shows the monoamine oxidase activity. Monoamine oxidase run alone, x—x—x; monoamine oxidase run in the presence of phosphatidylcholine, Δ—Δ—Δ; monoamine oxidase run in the presence of cardiolipin, ●--●--●. The lower part of the figure shows lipid P. Phosphatidylcholine run alone, x—x—x; phosphatidylcholine run in the presence of monoamine oxidase, Δ—Δ—Δ; cardiolipin run alone, o----o----o; cardiolipin run in the presence of monoamine oxidase, ●----●----●.

methyl ketone extraction procedure of Hollunger and Oreland (1970).

Interactions between Monoamine Oxidase and Phospholipids in Solution. The experiments described above show that when lipid-depleted mitochondria have bound acidic phospholipids they can bind soluble monoamine oxidase. The prior formation of phospholipid–protein complexes in solution is not necessary for the binding of the enzyme, but increases the amount of enzyme bound. We have therefore carried out experiments to study the formation of such soluble enzyme–phospholipid complexes. In one type of experiment the behavior of the enzyme on Sepharose gel filtration in the presence and absence of phospholipids was studied. Figure 5 shows that in the absence of phospholipids the enzyme was eluted as a single, included peak. The addition of phosphatidylcholine did not change the elution pattern of the enzyme activity whereas the addition of cardiolipin caused some of the enzyme activity to be eluted in the void volume. This shows that at least some of the enzyme had interacted with the cardiolipin.

In another type of experiment, the behavior in sucrose density gradient centrifugation of the enzyme and of phospholipid dispersions, alone or in combination, was studied. The enzyme alone gave a major peak in the middle of the gradient (Figures 6 and 7). Phosphatidylcholine alone was concentrated to the top of the gradient (Figure 6). When enzyme and phosphatidylcholine were run together, neither changed its place in the gradient. Cardiolipin run alone gave a peak in the upper part of the gradient, but when run in the presence of enzyme, the cardiolipin was found in the middle of the gradient coincident with the enzyme activity (Figure 6). The dispersion of phosphatidylinositol plus phosphatidylserine run alone was concentrated mainly in the upper part of the gradient (Figure 7). When the dispersion and enzyme were run together, phospholipid and enzyme changed their locations in the gradient and were both found mainly in the lower part. These results indicate that the enzyme interacted with the cardiolipin dispersion and with the dispersion of phosphatidylinositol plus phosphatidylserine, but did not interact with the phosphatidylcholine dispersions. This suggests that the enzyme can form soluble complexes with the same phospholipids that have been shown to bind it to lipid-depleted mitochondria (Table II).

Discussion

The essential conclusion from the present experiments is that the soluble monoamine oxidase obtained from lipid-depleted pig liver mitochondria can form complexes with several highly acidic phospholipids. In the presence of lipid-depleted mitochondria and of acidic phospholipids the enzyme re-bound to the mitochondria. Lipid-depleted red cell membranes and lipid-depleted milk fat globule membranes were as effective as lipid-depleted mitochondria, demonstrating that there was no high specificity for the nature of the insoluble membrane preparation. Sucrose density gradient and gel

filtration experiments gave evidence for formation of soluble enzyme–phospholipid complexes. On the other hand, the enzyme bound to relipidated mitochondrial residues without prior formation of such enzyme–phospholipid complexes. Thus, the enzyme can interact either with phospholipid in solution, forming soluble complexes, or with phospholipid bound to insoluble membrane residues, forming insoluble complexes. There are two ways in which this interaction may take place. The enzyme may pick up a certain number of phospholipid molecules to form a protein–phospholipid complex of definite structure. Alternatively, the enzyme may bind to the phospholipid micelles. Some evidence favors the latter possibility. In the density gradient experiments with cardiolipin, for instance, a large proportion of the phospholipid changed its position in the gradient in the presence of enzyme. If all the phospholipid that changed its distribution interacted with the enzyme, the complexes formed contained 90–95% phospholipid by weight, corresponding to more than 700 moles of cardiolipin/mole of enzyme. Likewise, the amount of phospholipid necessary to cause binding of enzyme to the lipid-depleted mitochondria was relatively large. No simple stoichiometry was apparent, but the lowest molar ratio of added cardiolipin to re-bound enzyme in our experiments was about 600.

The fact that only highly acidic phospholipids and not the zwitterionic phospholipids formed complexes with the enzyme or caused its binding to lipid-depleted membranes suggests that the charge of the phospholipid is important. This would be the case for instance if the binding was mainly electrostatic and involved positively charged groups on the protein and negative charges on the phospholipid. Such electrostatic binding of membrane proteins to acidic phospholipids has been demonstrated (Das and Crane, 1964; Quinn and Dawson, 1969) but our data are not compatible with this type of binding for monoamine oxidase. The isoelectric point of the enzyme is about 4.7 (L. Oreland, unpublished data) and it is thus negatively charged at pH 7.2. In agreement with this it did not bind to cation exchangers. It is therefore unlikely that the enzyme would bind electrostatically to anionic phospholipids but not to zwitterionic phospholipids. Furthermore, increased salt concentration only slightly decreased the extent of binding, and the rebound enzyme could not be extracted by a buffer with high salt concentration. Thus, we conclude that the interaction of the monoamine oxidase with the acidic phospholipids was not mainly by ionic bonds.

Further information on the nature of the binding could have been obtained if the binding had been tested at extremes of pH. This would have changed the dissociation of the phospholipid and the protein and would thus have allowed an evaluation of the importance of the charges. Chemical modification of the enzyme would also have been informative. However, these manipulations would have resulted in loss of enzyme activity. Our studies depend on measurement of enzyme activity since the enzyme preparation used was not pure. An alternate way of measuring the enzyme would have been to measure the amount of covalently bound flavine (Igaue *et al.*, 1967; Erwin and Hellerman, 1967; L. Oreland, unpublished data) in the supernatant or sediment. This has been used by other investigators in studies on mitochondrial succinic dehydrogenase (Singer *et al.*, 1962; Cerletti *et al.*, 1968). However, since mitochondria contain at least two major enzymes with covalently bound flavine, *i.e.*, succinic dehydrogenase and monoamine oxidase, such data may measure a mixture of these two enzymes.

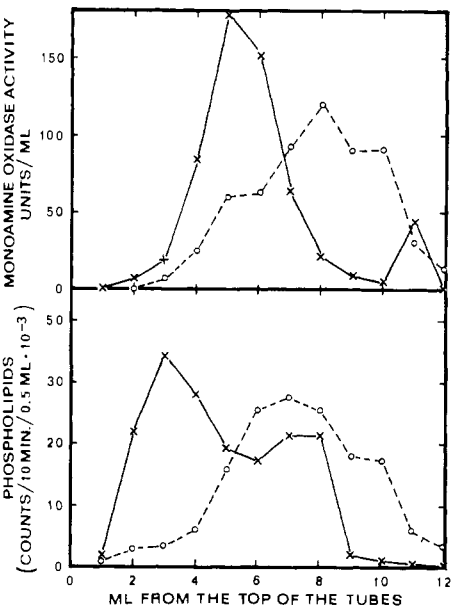

FIGURE 7: Sucrose density gradient centrifugation of soluble monoamine oxidase in the presence of dispersed phosphatidylinositol + phosphatidylserine. Sucrose density gradients were prepared in 3 tubes as described in the legend to Figure 6. In the tubes were layered in 1 ml of the 0.01 M phosphate buffer, respectively: 2 mg of [^{14}C]-phosphatidylinositol + [^{14}C]phosphatidylserine; buffer extract containing 650 units of soluble monoamine oxidase; and 2 mg of the phospholipids + 650 units of enzyme. The tubes were centrifuged at 120,000*g* for 16 hr at 4° in a Spinco rotor SW-40. The tubes were then fractionated as described in the legend to Figure 6. Monoamine oxidase activity was estimated as described in Methods and the phospholipids estimated as counts/10 min by a Packard liquid scintillation spectrometer Model 3320. Upper part of the figure: monoamine oxidase run alone, x—x—x; monoamine oxidase run in the presence of phosphatidylinositol + phosphatidylserine, o----o----o. Lower part of the figure: phosphatidylinositol + phosphatidylserine run alone, x—x—x; phosphatidylinositol + phosphatidylserine run in the presence of monoamine oxidase, o----o----o.

Several mitochondrial enzymes depend on phospholipids for their activity (for a review, see Rothfield and Finkelstein, 1968). We found that the presence or absence of added phospholipid did not affect the activity of monoamine oxidase in solution. It is therefore unlikely that interaction with phospholipids caused drastic changes in the conformation of the enzyme, but minor changes may occur. It is also possible that the soluble enzyme is already a protein–phospholipid complex (Erwin and Hellerman, 1967; Gomes *et al.*, 1969).

An unresolved question is whether the re-bound enzyme is back in its normal place in the mitochondria. Some evidence indicates that a correlation may exist. Thus, we have previously (L. Oreland and T. Olivecrona, to be published) shown that it is necessary to remove the highly acidic phospholipids from the mitochondria to render the enzyme buffer soluble. We now find that it is these phospholipids which are effective in rebinding the enzyme to the lipid-depleted mitochondria. Furthermore, the enzyme re-bound to the mitochondria by cardiolipin could not be extracted by buffer, in agreement with the properties of the enzyme in the intact mitochondria. However, since we do not know how the enzyme bound to the phospholipids, nor how the phospholipids bound to the lipid-depleted mitochondria, it is impossible to draw any conclusions on the situation *in vivo*.

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Incorporation of Branched-Chain C_6-Fatty Acid Isomers into the Related Long-Chain Fatty Acids by Growing Cells of *Bacillus subtilis**

Toshi Kaneda

ABSTRACT: The incorporation of eight C_6-alkanoic fatty acids into long-chain fatty acids by the growing cells of *Bacillus subtilis* (ATCC-7059) has been studied. Among them, five branched C_6-fatty acids are found to be incorporated into long-chain fatty acids and in each case a set of three new fatty acids are produced. The new fatty acids produced (3–24% of the total fatty acids) are 10-methyltridecanoic, 12-methylpentadecanoic, and 14-methylheptadecanoic from 2-methylpentanoic; 11-methyltridecanoic, 13-methylpentadecanoic, and 15-methylheptadecanoic from 3-methylpentanoic; 10,10-dimethyldodecanoic, 12,12-dimethyltetradecanoic, and 14,14-dimethylhexadecanoic from 2,2-dimethylbutyric; 11,11-dimethyldodecanoic, 13,13-dimethyltetradecanoic, and 15,15-dimethylhexadecanoic from 3,3-dimethylbutyric; 10-ethyldodecanoic, 12-ethyltetradecanoic, and 14-ethylhexadecanoic from 2-ethylbutyric. These are identified by gas–liquid chromatography and mass spectrometry using the structually related chemical C_{15}-fatty acids as standard. The major long-chain fatty acid is, in all cases, the C_{16}-fatty acid, and the two others, C_{14}- and C_{18}-fatty acids, are produced in much smaller proportions. This fatty acid distribution is very similar to that obtained when C_2- and C_4-fatty acids are incorporated into long-chain fatty acids. Thus it is concluded that the chain length of fatty acids achieved in the synthesis is not affected by the chain length or nature of chain initiator used: the C_{16}-fatty acids being always the major fatty acids regardless of whether C_2-, C_4-, or C_6-fatty acid is used. The relative activity of the five active C_6-fatty acids as the chain initiator is 2-ethylbutyric > 2-methylpentanoic > 3-methylpentanoic > 2,2- or 3,3-dimethylbutyric. Apparently C_6-fatty acids having single branching (either methyl or ethyl) at α position are excellent chain initiators. The other C_6-fatty acids tested but found to give no increase in expected or related long-chain fatty acids are caproic, isocaproic, and 2,3-dimethylbutyric.

Synthesis (*de novo*) of long-chain alkanoic acids has been shown to include the repeated condensation of the chain extender, C_2 precursor, with the chain initiator to extend its chain length to 12–18 carbon atoms. The chain extender is, in all known systems, malonyl-CoA and no exception has been reported (Brady, 1958; Wakil, 1958; Vagelos, 1964).

The chain initiator, however, could be one of several acyl-CoA esters. In the majority of organisms, acetyl-CoA serves as chain initiator and the main product is palmitic acid. In some organisms, on the other hand, branched-chain acyl-CoA esters, namely, isobutyryl, α-methylbutyryl, and iso-

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