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Relationship Between Losses in Cytochrome Oxidase Activity and Peroxidation of Monosaturated Phosphatidylcholines and Phosphatidylethanolamines

E. Santiago, Natalia López-Moratalla * and Josefa L. Segovia *

Departamento de Bioquímica
Universidad de Navarra
Pamplona (España)

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Incubation of inner mitochondrial membranes from rat liver in the presence of inducers of peroxidation reactions, such as ascorbate or cysteine, produced a large loss in cytochrome oxidase activity parallel to the disappearance of phosphatidylcholine and phosphatidylethanolamine molecular species, which contained a saturated and an unsaturated fatty acid. The loss in enzyme activity was unrelated to alterations in other species of these phospholipids or other ones. These results may reflect the existence of specific associations within the membrane between cytochrome oxidase and monosaturated phosphatidylcholines and/or phosphatidylethanolamines.

There seems to be no doubt regarding the existence of *in vivo* associations of cytochrome oxidase with phospholipids, since cytochrome oxidase preparations contain phospholipids, and besides the removal and addition of lipids influence its activity. However, and despite the large number of reports on this matter, no unanimous view has been reached so far with respect to the specificity of the phospholipid associated with this enzyme in animal tissues or in yeasts (1, 2, 4-6, 8, 13, 14, 23, 24). The existence of associations between mitochondrial ATPase from rat liver and linoleylcardiolipins has been proposed (11, 17) after having observed that there is a linear relationship between their selectivie peroxidation and the loss of ATPase activity. The loss in activity of certain membrane bound enzymes as a result of peroxidation reactions had already been reported (3, 10, 15, 22). SANTIAGO *et al.* (20) have shown that, in peroxidation reactions induced on mitochondrial membranes by ascorbate or cysteine, a decrease

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in extractable phospholipids takes place due to alterations of the unsaturated fatty acids present in their molecules. The peroxidized phospholipids become then strongly bound to membrane proteins and cannot be extracted by the usual mixtures of organic solvents. Forming part of the altered molecules of these phospholipids bound to the protein, one may find saturated fatty acids, which of course cannot be peroxidized, and some of the unsaturated fatty which might have escaped the peroxidation reaction (19).

The disappearance of extractable phospholipids and of their fatty acid moieties along the incubation of membranes in the presence of peroxidation inducers, as well as the recovery of certain fatty acids in the degraded phospholipids bound to the protein, together with the decrease in activity of cytochrome oxidase, have been studied.

The results presented in this report show that a parallelism exists between losses in cytochrome oxidase activity and peroxidation of phosphatidylcholine and phosphatildylethanolamine containing in their molecules one saturated and one unsaturated fatty acid; these data might very well indicate the presence within the membrane of specific associations between cytochrome oxidase and those phospholipids.

Materials and Methods

Male Wistar rats weighing approximately 200 g were used in all the experiments. Liver mitochondria were prepared according to the method of HOGEBOOM (9). Isolated mitochondria were subjected to osmotic rupture following the method of PARSONS *et al.* (16). Inner mitochondrial membranes were obtained using Parsons «low speed pellet» as starting material. In order to remove the outer membranes still present in this fraction, it was thoroughly washed three times by resuspending it in 0.02 M phosphate buffer, pH 7.4, centrifuging at 1,900 × *g* for 15 minutes, and once more resuspending it in 0.25 M sucrose and centrifuging at 8,500 × *g* for 10 minutes (20). Incubations of mitochondrial membranes were carried out in a medium, 1 mM ascorbate, or 0.8 mM cysteine, 0.02 Tris-HCl buffer, pH 7.4, and 0.25 M sucrose, at 30° C, for 60 or 120 minutes respectively, with continous stirring. Controls without ascorbate or cysteine were incubated simultaneously. The amount of inner membranes present in the medium was adjusted to give a final concentration of 0.8 mg protein/ml. The lysis of the membranes was followed by determining the absorbance changes at 520 nm, using cuvettes with 1 cm light path. Aliquots were taken at different intervals to carry out the determinations of lipids and enzyme activity.

Proteins were determined by the method of LOWRY *et al.* (12). Phospholipids were extracted as previously described (18) from 10 ml aliquots of the membrane suspensions after precipitation with enough conc. $HClO_4$ to give a final concentration of 0.3 N; the pellets obtained after centrifugation were resuspended in 5 ml of a solvent described by FOLCH (7) which consisted of chloroform-methanol-conc. HCl (200:100:1). After standing at room temperature for 30 minutes the extracts were washed with 5 ml of cold 0.1 N HCl, centrifuged, and the bottom chloroform layer carefully removed. The protein interface was extracted once more and the chloroform layers pooled. Phospholipids were separated by thin layer chromatography according to the technique of NESKOVIC *et al.* (12). Lipid phosphorus was determined by the method of BARTLETT (18). Methyl esters of the fatty acids present in each phospholipid were prepared by direct transmetylation catalyzed by BF_3 as described by MORRISON and SMITH (7); methyl esters were then analyzed with a Beckman GC4 gas chromatograhp, using a double column, with a 1/8 inch diameter and a 6 ft length; the liquid phase was

20 % DEGS, and the solid phase, Chromosorb W; particle size, 24/60 μ diameter; hydrogen and air flows were respectively 50 cc/min and 250 cc/min; column temperature, 160° C and that of the detectors, 280° C.

Methylation of fatty acids present in the altered phospholipids still bound to proteins, after repeated lipid extraction, was carried out as follows: 6 ml of 96 % ethanol and 0.4 ml of 10 N NaOH were added to the protein precipitate amounting up to 20 mg, and heated in a water bath during 30 minutes, checking that the medium remained alkaline. The hydrolyzate was acidified with HCl and the fatty acids extracted with 3 ml of petroleum ether (b.p. 60° C) and methyl esters prepared as described above.

Cytochrome oxidase activity was determined by the method of SOTTOCASA *et al.* (21). The interference of ascorbate in the determination of cytochrome oxidase activity was eliminated with ascorbate oxidase as previously described (10).

Results and Discussion

Aliquots from the incubation mixture were taken at different intervals and lipid extracts prepared and chromatographed; the separated phospholipids remaining at those intervals were analyzed and the fatty acids present in each one of them determined. The phosphorus corresponding to the altered phospholipids, which was tightly bound to the protein, as well as the fatty acids present in them, were determined in the protein precipitates, once the extractable lipids had been removed. Cytochrome oxidase activity was also measured at the same intervals.

No losses in cytochrome oxidase activity nor in extractable phospholipids were observed during the incubation of the controls. No changes in absorbancy were

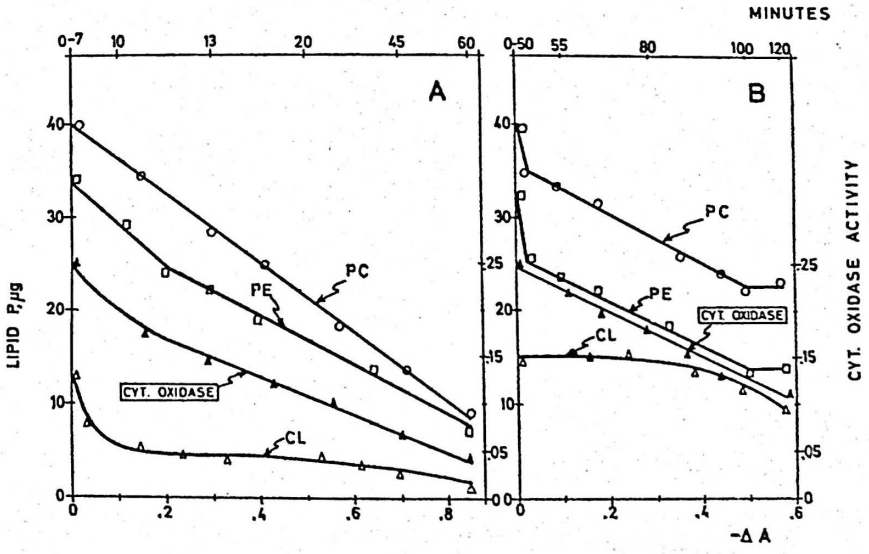

Fig. 1. *Changes in absorbance (—ΔA), phospholipid and cytochrome oxidase activity during incubation of isolated rat liver inner mitochondrial membranes in the presence of ascorbate (A) or cysteine (B).*

The values of lipid P corresponding to each phospholipid are referred to 20 mg membrane protein. Cytochrome oxidase activity is expresed as μmoles $\times$ min^{-1} $\times$ mg^{-1}. Each point represents the average of 5 independent experiments. PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; CL: Cardiolipin.

observed in the control suspension during the incubation.

The loss in cytochrome oxidase activity, as well as the decrease in the different phospholipids present in the lipid extracts, have been plotted against absorbance changes at different intervals during the incubation of inner mitochondrial membranes in the presence of ascorbate figure 1A) or cysteine (figure 1B). It was found convenient to use $-\Delta A$ as linear variable rather than time because of the more exact reproducibility in different experiments with respect to the parameters studied; the lag preceding the changes related to the peroxidation reaction varied at times within certain limits. It may be observed that whereas a close parallelism exists between the inactivation of cytochrome oxidase and the decrease of extractable phosphatidylethanolamine and phosphatidylcholine, both in incubations with ascorbate or with cysteine, this parallelism does not exist in neither case with respect to cardiolipin.

The fatty acid composition of each phospholipid present in the lipid extracts was also followed along the incubation time. In figures 2 and 3, the percent decrease in cytochrome oxidase activity has been plotted against the percent disappearance of the different fatty acids of phosphatidylethanolamine and of phosphatidylcholine during the incubation of the membranes in the presence of ascorbate or cysteine. In all cases, a straight correlation exists beteen the disappearance of saturated fatty acids and the decrease in cytochrome oxidase activity; this would be an indication that the molecular species of these phospholipids affecting the activity of cytochrome oxidase contain one saturated fatty acid; this fatty acid must be accompanied by an unsaturated fatty acid, since the disappearance of the saturated fatty acids is necessarily mediated by

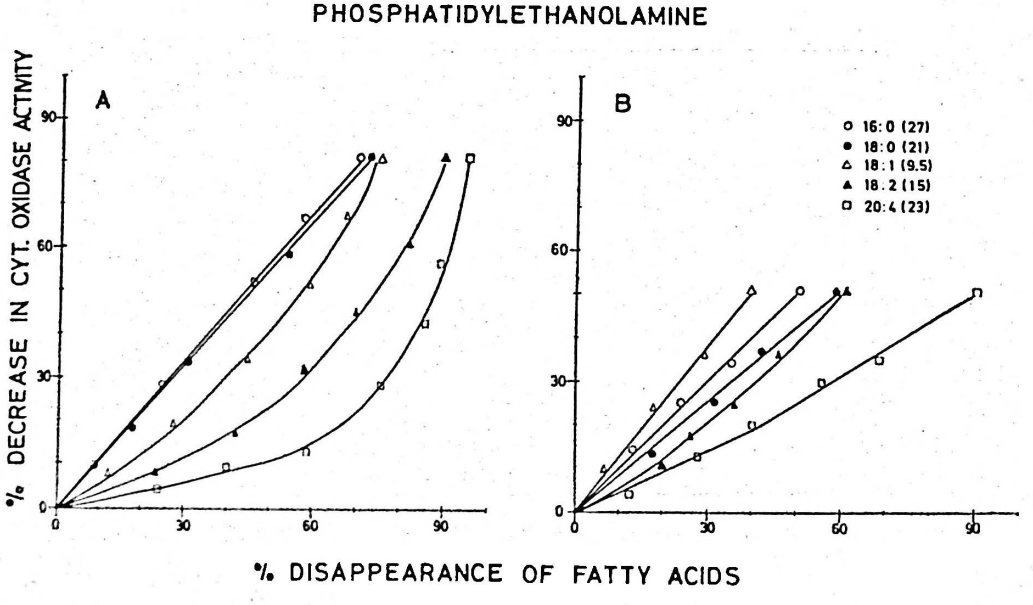

Fig. 2. *Correlation between percent decrease in cytochrome oxidase activity and percent disappearance of fatty acids in phosphatidylethanolamine during the incubation of inner mitochondrial membranes in the presence of either ascorbate (A) or cysteine (B).* Each point represents the average of 5 independent experiments. In parentheses the μg of each fatty acid per mg of protein have been indicated.

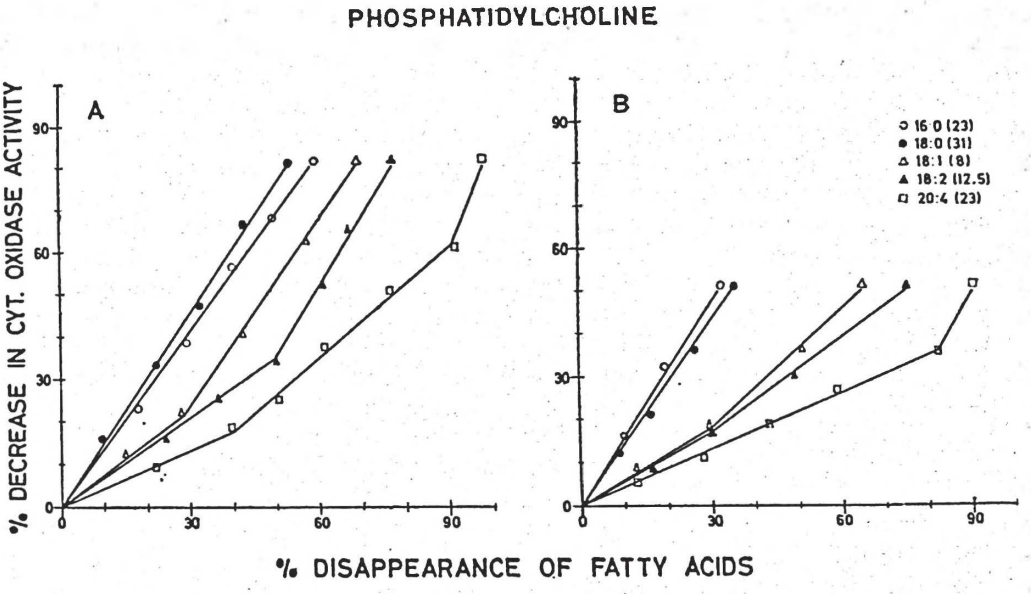

Fig. 3. *Correlation between percent decrease in cytochrome oxidase activity and percent disappearance of fatty acids in phosphatidylcholine during the incubation of inner mitochondrial membranes in the presence of either ascorbate (A) or cysteine (B).*
Each point represents the average of 5 independent experiments. In parentheses the μg of each fatty acid per mg of protein have been indicated.

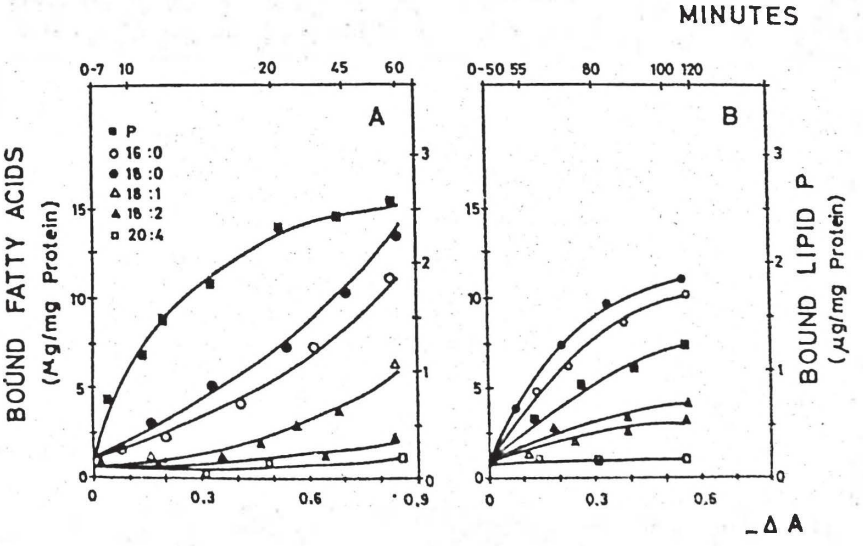

Fig. 4. *Fatty acids and lipid phosphorus bound to protein during the incubation of inner mitochondrial membranes in the presence of ascorbate (A) or cysteine (B).*
Fatty acids and lipid P are expresed as $\mu g \times mg^{-1}$ protein.

the presence in the same phospholipid molecule of an unsaturated fatty acid which is the moiety susceptible of peroxidation. The amounts of the different fatty acids and of lipid phosphorus, which became bound to protein along the incubation, have been represented in figures 4A and 4B. It may be observed that in the early stages after the onset of the peroxidation induced by ascorbate a large amount of bound lipid P appeared as compared to the amount of bound fatty acids. This may indicate that the molecular species of phospholipids mainly affected in the early stages of the peroxidation reaction would be those with only unsaturated fatty acids, which are consequently destroyed and thus giving rise to an unparallelled increase in P. Later on, and after the disappearance of those unsaturated species, the progressive binding of saturated fatty acids to protein corresponds to molecular species of phospholipids with saturated and unsaturated fatty acids. However, the graphs representing the lipid P and fatty acids bound to protein during the incubation with cysteine (fig. 4B) suggest that in this case the molecular species with only unsaturated fatty acids were not preferently affected by the peroxidation reaction, as it happened when this took place in the presence of ascorbate (fig. 4A).

That the molecular species of phosphatidylethanolamine and phosphatidylcholine with two unsaturated fatty acids do not affect cytochrome oxidase activity is clearly reflected by the fact that in the case of the incubation with ascorbate the slope of the initial part of the curve, corresponding to the different unsaturated fatty acids, was much less steep (figs. 2A and 3A). This part of the curve corresponds to the degradation both of the species with two unsaturated fatty acids and of those with one saturated and one unsaturated fatty acid. After the nearly complete degradation of the molecular species with two unsaturated fatty acids, the remaining unsaturated fatty acids which still continue disappearing correspond predominantly to the species of one saturated and one unsaturated fatty acid; the slope becomes then steeper, being this a new proof of the dependence of cytochrome oxidase activity from these latter species.

During the incubation with cysteine a correlation between loss of cytochrome oxidase activity and the disappearance of unsaturated fatty acids in phosphatidylethanolamine and phosphatidylcholine has been found (figs. 2B and 3B); there is no change in slope, because the molecular species with two unsaturated fatty acids were not preferently affected in the early stages after the onset of the peroxidation, as it was the case when the incubation was carried out in the presence of ascorbate.

The results suggest the existence of specific associations of cytochrome oxidase with monosaturated species of phosphatidylcholine and/or phosphatidylethanolamine. This association as well as that already reported between ATPase and cardiolipins (17) containing exclusively linoleic acid (11), would imply the existence of a marked microheterogeneity within the inner mitochondrial membrane.

Resumen

La incubación de membranas internas mitocondriales de hígado de rata en presencia de agentes inductores de reacciones de peroxidación, tales como el ascorbato o la cisteína, origina una gran pérdida de la actividad citocromo oxidasa paralela a la desaparición de las especies moleculares de fosfatidilcolina y fosfatidiletanolamina que contienen un ácido graso saturado y otro insaturado. Las pérdidas de la actividad enzimática no guardaron relación con las alteraciones de otras especies de éstos o de otros fosfolípidos. Estos resultados pueden reflejar la existencia de asociaciones específicas en la membrana entre la citocromo oxidasa y las fosfatidilcolina y/o fosfatidiletanolamina monosaturadas.

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