REVISTA ESPAÑOLA DE FISIOLOGIA, 29, 329-334. 1973

# Specificity of Association Between Linoleylcardiolipins and Mitochondrial ATPase

**N. López-Moratalla, J. L. Segovia and E. Santiago**

Department of Biochemistry
University of Navarra
Pamplona (Spain)

(Received on September 29, 1973)

N. LOPEZ-MORATALLA, J. L. SEGOVIA and E. SANTIAGO. *Specificity of Association Between Linoleylcardiolipins and Mitochondrial ATPase.* Rev. esp. Fisiol., 29, 329-334. 1973.

Mitochondrial ATPase activity decreases during the incubation of rat liver inner mitochondrial membranes in the presence of ascorbate or cysteine, due to alteration of membrane phospholipids through peroxidation reactions. A straight correlation between decrease in mitochondrial ATPase activity and the alteration of the molecular species of cardiolipins containing solely linoleic acid has been found. This correlation does not exist with other molecular species of cardiolipin or any other phospholipids present in the membrane. These results support a specificity of association between mitochondrial ATPase and linoleyl cardiolipins.

It has already been established that phospholipids present in mitochondrial membranes are degraded through peroxidation of their unsaturated fatty acid constituents, when the membranes are incubated with either ascorbate or cysteine. The altered phospholipids remain strongly bound to the neighboring proteins and cannot be extracted with the usual organic solvent mixtures (17). These phospholipid alterations result in a decrease, or even in the complete loss of activity of a number of membrane bound enzymes (7). It has been shown in a previous report (18) that, during the incubation of rat liver inner mitochondrial membranes in the presence of peroxidation inducing reagents, a close correlation exists between the progressive decrease in ATPase activity and the amount of cardiolipin being simultaneously degraded. No relationship was found between the loss of activity of this enzyme and the degradation of other phospholipids. These results suggest a specific association of the enzyme ATPase with cardiolipin. Since cardiolipin can exist in different molecular species, being predominant the species containing solely linoleic acid (6, 20), it was decided to investigate further if the specificity of association between ATPase and cardiolipin would also involve the acyl residues of this phospholipid. The results here reported support such specificity of association between mitochondrial ATPase and linoleylcardiolipins.

## Materials and Methods

Male Wistar rats weighing approximately 200 g were used in all the experiments. Livers were homogenized in 0.25 M sucrose and mitochondria isolated according to the method of HOGEBOOM (3). Inner mitochondrial membranes were prepared according to the method of PARSONS *et al.* (13) with the modifications already described (16). Incubation of inner mitochondrial membranes were carried out in a medium containing 1 mM ascorbate or $8 \times 10^{-4}$ M cysteine, 0.02 M Tris-HCl buffer, *p*H 7.4 and 0.25 M sucrose, at 30° C. Controls without ascorbate or cysteine were incubated simultaneously. Inner membranes resuspended in 0.25 M sucrose were added to the incubation medium to give an approximate concentration of 0.8 mg of protein per ml. Proteins were determined by the method of LOWRY *et al.* (8). Lipids were extracted from the sediment obtained after precipitating the proteins with enough conc. $HClO_4$ to give a final concentration of 0.3 N, as previously described (15). Phospholipids were separated by thin layer chromatography as described by NESKOVIC *et al.* (10). Lipid phosphorus was determined as described by BARTLETT (1) after wet ashing. Methyl esters of the fatty acids of the different phospholipids present in the lipid extract were prepared through direct methylation with 14 % $BF_3$ in methanol according to MORRISON and SMITH (9). Methyl esters were then analyzed with a Beckman GC-4 gas chromatograph, 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, 42/60 $\mu$ diameter; hydrogen and air flow were respectively 60 cc/min and 250 cc/min; column temperature, 160° C and that of the detectors, 280° C. ATPase activity was determined by the method of PULLMAN *et al.* (14).

## Results and Discussion

The precent distribution, in terms of lipid phosphorus, for the major phospholipids found in the inner mitochondrial membranes was: phosphatidylcholine, 40 %; phosphatidylethanolamine, 33 %; and cardiolipin, 15 %. The distribution of

Table I. *Percent distribution of fatty acids in total phospholipid extract, and in phosphatidylethanolamine, phosphatidylcholine and cardiolipin.*
< 16, Fatty acids with less than 16 C. The number of double bonds in 17:x fatty acids has not been determined. Number of experiments, 10.

| Fatty acids | Total | Phospholipids* | | |
|---|---|---|---|---|
| | | PC | PE | CL |
| < 16 | 0.4 ± 0.1 | 0.2 ± 0.1 | 0.4 ± 0.1 | 0.3 ± 0.1 |
| 16 : 0 | 19 ± 1 | 23 ± 1 | 26 ± 1 | 8 ± 1.5 |
| 16 : 1 | 2 ± 0.5 | 1 ± 0.3 | 1.6 ± 0.4 | 3 ± 1 |
| 17 : 0 | 0.6 ± 0.1 | 0.5 ± 0.1 | 0.7 ± 0.2 | 0.4 |
| 17 : x | 0.2 ± 0.1 | 0.3 | 0.3 ± 0.4 | 0.1 |
| 18 : 0 | 22 ± 1 | 30 ± 1 | 21.5 ± 1.5 | 10 ± 1 |
| 18 : 1 | 10 ± 1 | 8.7 ± 1 | 9.5 ± 1 | 19 ± 2 |
| 18 : 2 | 22.2 ± 1 | 12.5 ± 1.5 | 15 ± 1 | 45 ± 2 |
| 20 : 2 + 20 : 3 | 2.2 ± 1 | 2 ± 1 | 3 ± 1 | 6 ± 4 |
| 20 : 4 | 21 ± 2 | 23 ± 1 | 23 ± 1 | 9 ± 1 |

* Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin; data represent mean ± S.D.

the different fatty acids in each phospholipid is given in Table I. All these values are in agreement with data previously reported in the literature (2, 4, 11, 12, 19).

The changes in ATPase activity which took place during the incubation of inner mitochondrial membranes in the presence of either ascorbate or cysteine expressed as percent inactivation, have been plotted in Figures 1, 2, 3 and 4 against the percent disappearance of the fatty acids present in the total lipid extract as well as in the

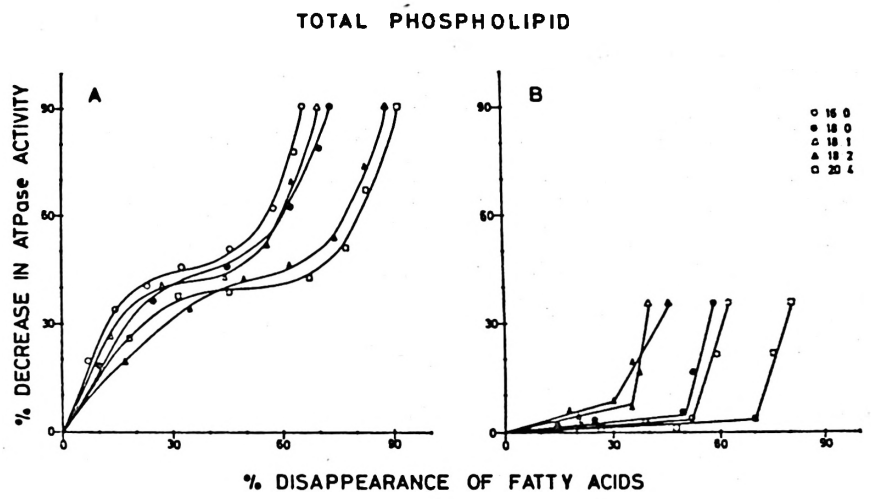


Fig. 1. *Correlation between percent decrease in ATPase activity and percent disappearance of fatty acids in the total lipid extract, along the incubation of inner mitochondrial membranes in the presence of either ascorbate (A) or cysteine (B).*
Each point represent the average of 5 indepent experiments.

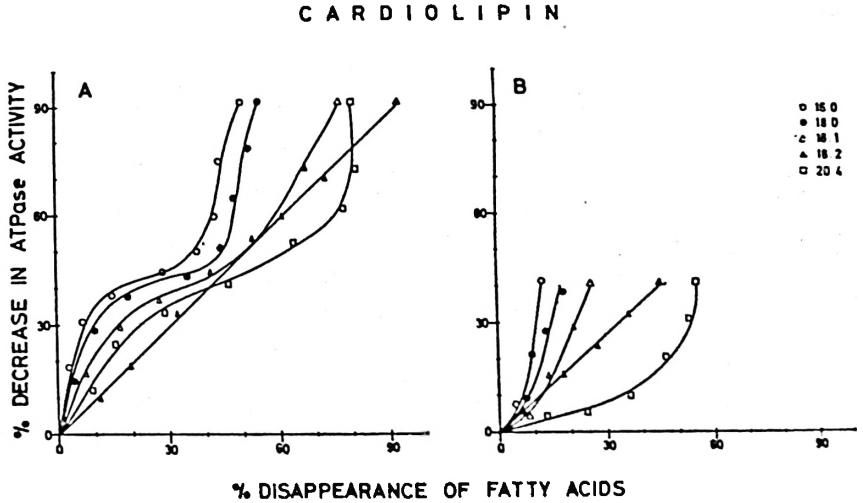


Fig. 2. *Correlation between percent decrease in ATPase activity and percent disappearance of fatty acids in the cardiolipin, along the incubation of inner mitochondrial membranes in the presence of either ascorbate (A) or cysteine (B).*
Each point represent the average of 5 indepent experiments.

individual phospholipids, cardiolipin, phosphatidylethanolamine and phosphatidylcholine. No correlation whatsoever can be found between decrease of ATPase activity and losses of each fatty acid in the total lipid extract. Correlation was also lacking with any of the fatty acid losses in the isolated phosphatidylethanolamine and phosphatidylcholine. However, when the decrease in ATPase activity is plotted against the percent degradation of the individual fatty acids present in cardiolipin, at different intervals during the incubation of the membranes with cysteine or ascor-

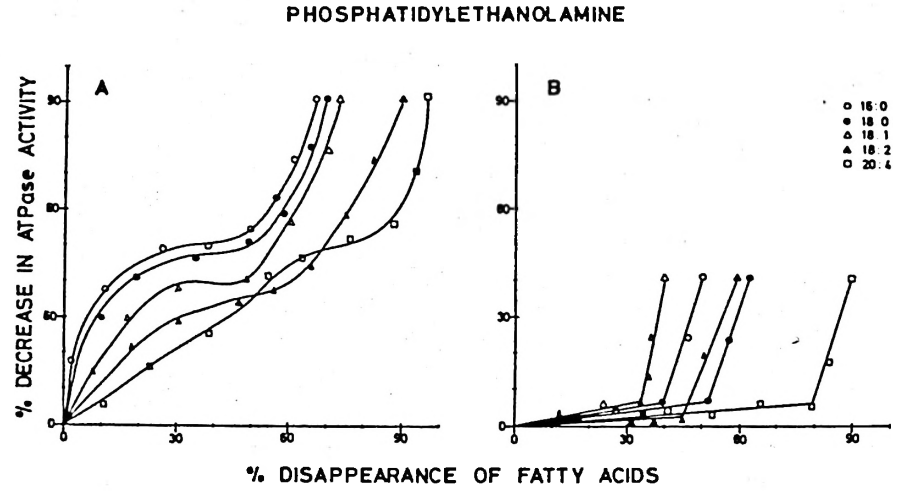


Fig. 3. *Correlation between percent decrease in ATPase activity and percent disappearance of fatty acids in the phosphatidylethanolamine, along the incubation of inner mitochondrial membranes in the presence of either ascorbate (A) or cysteine (B).* Each point represent the average of 5 indepent experiments.

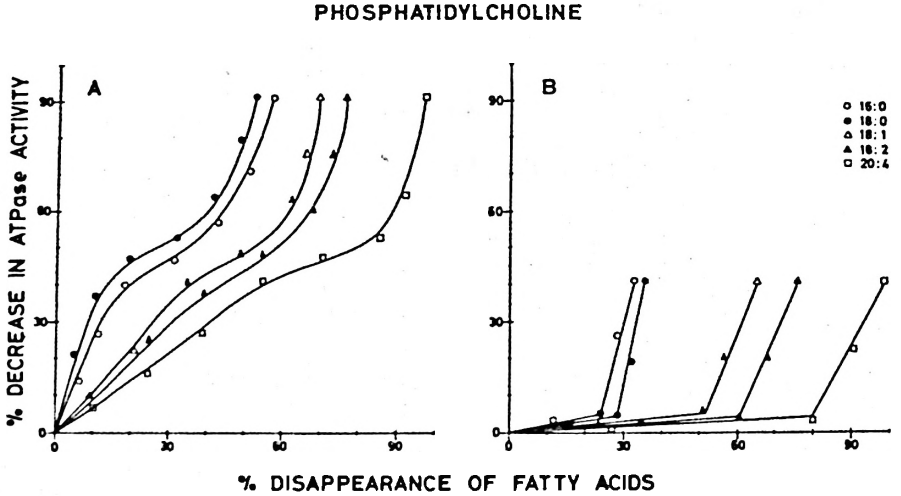


Fig. 4. *Correlation between percent decrease in ATPase activity and percent disappearance of fatty acids in the phosphatidylcholine, along the incubation of inner mitochondrial membranes in the presence of either ascorbate (A) or cysteine (B).* Each point represent the average of 5 indepent experiments.

bate, the existence of a clear correlation between the decrease in ATPase activity and degradation of linoleic acid is obvious. This correlation is absent with respect to other fatty acids of the cardiolipins.

These results can be interpreted as an evidence that mitochondrial ATPase is associated only with molecular species of cardiolipin containing exclusively linoleic acid. The results presented here are consistent with the observations made by KAGAWA and RACKER (5) that linoleic acid reactivated beef heart mitochondrial ATPase preparations low in phospholipids. KAGAWA and RACKER have also shown that linoleic acid alone could not restore the oligomycin sensitivity; only individual phospholipids or lipid mixtures could restore the olygomycin sensitivity of the ATPase preparation. In view of the data presented herein it is very posible that the molecular species of cardiolipin containing solely linoleic acid would be specific at the same time to reactivate both the enzymic activity and the oligomycin sensitivity of the delipidated ATPase preparations.

*ACKNOWLEDGEMENTS*

This work was supported by a grant «Ayuda a la Investigación en la Universidad (Ministerio de Educación y Ciencia)». N. L.-M. and J. L. S. were recipients of fellowships from «Consejo Superior de Investigaciones Científicas» and «Ministerio de Educación y Ciencia» respectively.

## Resumen

Durante la incubación de membranas mitocondriales en presencia de ascorbato o cisteína se produce un marcado descenso de la actividad de la ATPasa mitocondrial, debido a alteraciones de los fosfolípidos causados por reacciones de peroxidación. Se ha encontrado una correlación estrecha entre el descenso de la actividad mitocondrial y la alteración de las especies moleculares de las cardiolipinas que contienen exclusivamente ácido linoleico. Esta correlación no existe con otras especies moleculares de cardiolipina o con cualquiera de los otros fosfolípidos presentes en la membrana. Estos resultados apoyan la existencia de una especificidad de asociación entre la ATPasa mitocondrial y linoleilcardiolipinas.

## References

1. BARTLETT, G. R.: *J. Biol. Chem.*, 234, 466, 1959.
2. COLBEAU, A., NACHAUR, J. and VIGNAIS, P. M.: *Biochim. Biophys. Acta*, 249, 462, 1971.
3. HOGEBOOM, G. H.: In «Methods in Enzymology» (S. I. COLOWICK and N. O. KAPLAN, eds.), vol. I, Academic Press, New York, 1955, p. 16.
4. HUET, C., LEVY, M., PASCAUD, M.: *Biochim. Biophys. Acta*, 150, 524, 1968.
5. KAGAWA, Y. and RACKER, E.: *J. Biol. Chem.*, 241, 2467, 1966.
6. KEENAN, T. W., AWASTHI, Y. C. and CRANE, F. L.: *Biochem. Biophys. Res. Comm.*, 40, 1102, 1970.
7. LÓPEZ-MORATALLA, N. and SANTIAGO, E.: *Rev. Med. Univ. Navarra*, 16, 127, 1972.
8. LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L. and RANDALL, R. J.: *J. Biol. Chem.*, 193, 265, 1951.
9. MORRISON, W. R. and SMITH, L. M.: *J. Lipid. Res.*, 6, 600, 1964.
10. NESKOVIC, N. M. and KOSTIC, D. M.: *J. Chromatog.*, 35, 297, 1968.
11. NEWMAN, H. A. I., GORDESKY, S. E., HOPPEL, C. and COOPER, C.: *Biochem. J.*, 107, 381, 1968.
12. PARKES, J. G. and THOMPSON, W.: *Biochim. Biophys. Acta*, 196, 162, 1970.
13. PARSONS, D. F., WILLIAMS, G. R. and CHANCE, B.: *Ann. N. Y. Acad. Sci.*, 137, 643, 1966.
14. PULLMAN, M. E., PENEFSKY, H. S., GATTA, A. and RACKER, E.: *J. Biol. Chem.*, 235, 3322, 1960.
15. SANTIAGO, E., MULÉ, S., REDMAN, C. M., HOKIN, M. R. and HOKIN, L. E.: *Biochim. Biophys. Acta*, 84, 550, 1964.
16. SANTIAGO, E., GANSER, A., MACARULLA, J. M. and GUERRA, F.: *Rev. esp. Fisiol.*, 24, 34, 1968.

17. SANTIAGO, E., VÁZQUEZ, J. J., EUGUI, J., MACARULLA, J. M. and GUERRA, F.: In «Membranes, Structure and Function» (J. R. VILLANUEVA and F. PONZ, eds.), Academic Press, London-New York, 1970, p. 17.
18. SANTIAGO, E., LÓPEZ-MORATALLA, N. and SEGOVIA, J. L.: *Biochem. Biophys. Res. Comm.*, **53**, 439, 1973.
19. STOFFEL, W. H. G.: *Z. Physiol. Chem.*, **234**, 1017, 1968.
20. WOOD, R. and HARLOW, R. D.: *Arch. Biochim. Biophys.*, **135**, 272, 1969.