Phosphatidic Acid Synthesis in Mitochondria

TOPOGRAPHY OF FORMATION AND TRANSMEMBRANE MIGRATION*

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The topography of formation and migration of phosphatidic acid (PA) in the transverse plane of rat liver mitochondrial outer membrane (MOM) were investigated. Isolated mitochondria and microsomes, incubated with sn-glycerol 3-phosphate and an immobilized substrate palmitoyl-CoA-agarose, synthesized both lyso-PA and PA. The mitochondrial and microsomal acylation of glycerophosphate with palmitoyl-CoA-agarose was 80–100% of the values obtained in the presence of free palmitoyl-CoA. In another series of experiments, both free polymyxin B and polymyxin B-agarose stimulated mitochondrial glycerophosphate acyltransferase activity approximately 2-fold. When PA loaded mitochondria were treated with liver fatty acid binding protein, a fifth of the phospholipid left the mitochondria. The amount of exportable PA reduced with the increase in the time of incubation. In another approach, PA-loaded mitochondria were treated with phospholipase A2. The amount of phospholipase A2-sensitive PA reduced when the incubation time was increased. Taken together, the results suggest that lysophosphatidic acid (LPA) and PA are synthesized on the outer surface of the MOM and that PA moves to the inner membrane presumably for cardiolipin formation.

Phosphatidic acid (PA),1 the key intermediate in the biosynthetic pathway of glycerolipids, is synthesized by two successive acylations of glycero-3-phosphate (1). The acylation steps are carried out by glycerophosphate acyltransferase (GAT) (2) and monoacylglycerophosphate acyltransferase (MGAT) (3). In mammalian cells, these enzymes are located in both MOM and endoplasmic reticulum (4–6). A substantial amount of knowledge has accumulated on mitochondrial GAT regarding its properties (7, 8), purification (9, 10), and cloning (11, 12). On the other hand, very little is known about MGAT.

Our previous work suggests that acyl-CoA synthetase, which activates fatty acids, spans the MOM (24). By using desulfo-CoA-agarose, an immobilized competitive inhibitor, we established that the catalytic site of the enzyme is located in the outer aspect of the MOM. The acyl-CoA formed can be used either catalytically for β-oxidation of the fatty acids in the mitochondrial matrix or can be used anabolically to acylate glycerophosphate. The synthesis of acyl-CoA on the outer surface of the MOM leads to the question: which leaflet of the MOM is involved in the synthesis of LPA and PA?

This paper deals with the location of synthesis of LPA and PA in the transverse plane of the MOM and the extent of translocation of PA necessary for cardiolipin synthesis. Our result suggests that both LPA and PA are synthesized on the outer aspect of the MOM, and subsequently, PA moves to the inner membrane as a precursor of cardiolipin.

EXPERIMENTAL PROCEDURES

Materials—Male Harlan Sprague-Dawley rats were purchased from Taconic Farms, Germantown, NY. sn-[2-3H]Glycerol 3-phosphate (1.11 × 104 cpm/nmol) was obtained from American Radiochemicals Inc. Palmitoyl-CoA-agarose, and polymyxin B-agarose, obtained from Sigma, were washed four times with 40 ml MTG buffer (MES/TEA/glycylglycine), pH 7.5, before use. All other materials were obtained as described previously (23).

Preparation of Mitochondria and Microsome—Liver mitochondria and microsome were prepared from 175–200-g male Harlan Sprague-Dawley rats as described previously (4). The purity of both preparations was evaluated by performing GAT assay in the presence and absence of 2 mM N-ethylmaleimide, which is an inhibitor of the microsomal GAT (15). Cross-contamination between mitochondrial and microsomal fractions was <3%.

Analytical Methods—GAT activity was measured by following the incorporation of sn-[2-3H]glycerol 3-phosphate into butanol-extractable phospholipids (15). Asolectin was omitted from the system. The concen-
tration of the subcellular protein in the incubation medium was main-
tained between 0.2 and 0.4 mg/ml. For sedimenting mitochondria or
microsomes, the incubated mixture was cooled to 0 °C and spun at
10,000 × g for 15 min or at 105,000 × g for 60 min, respectively. The
pellet was resuspended in 0.5 ml of water. The supernatant, the resus-
pended pellet, or whole GAT reaction mixture was treated with 1-but-
tanol to extract the radioactive acylation products, LPA and PA, which
were separated by thin layer or high performance thin layer chromo-
matography. Protein concentration was assayed as per the Bradford
method (25) using bovine serum albumin as a standard.

RESULTS

Activity of Mitochondrial and Microsomal GAT Using Palmi-
toyl-CoA-Agarose as Acyl Donor—Palmitoyl-CoA is commer-
cially available cross-linked to 4% beaded agarose. As the link-
age of agarose is with the amino group of CoA with a 7-carbon
spacer, it is not possible that the activated acyl group can cross
the MOM phospholipid bilayer and reach the inner aspect of
the membrane. Microsomal GAT has its catalytic site on the
outer surface of the membrane (7) and was used as a positive
control. Fig. 1 documents the activity of mitochondrial and
microsomal GAT at different concentrations of palmitoyl-CoA
and palmitoyl-CoA-agarose. For both mitochondria and micro-
somes, their activities of GAT is over 90% in the presence of
immobilized substrate when compared with the activities in
presence of free palmitoyl-CoA. Since the concentration of
palmitoyl-CoA in the immobilized sample cannot be accurately
measured, comparison can be made between the activities in
the presence of free and bound palmitoyl-CoA at their optimal
level of activity.

The palmitoyl-CoA-agarose was washed four times before

FIG. 1. Comparison of the optimal activity of mitochondrial
(■) and microsomal (□) GAT using palmitoyl-CoA (A) and palmi-
toyl-CoA-agarose (B) as acyl donor. The reaction was initiated by
the addition of the subcellular fraction. The final protein concentra-
tion was adjusted to 0.2 mg/ml for both mitochondria and microsomes.
The values are the average of two separate sets of experiments.

FIG. 2. Differential action of polymyxin B and polymyxin B-
agarose on mitochondrial (■) and microsomal (□) GAT. The
assays were performed at the optimal concentrations of palmitoyl-CoA
and palmitoyl-CoA-agarose for the subcellular fractions. Different con-
centrations of free and immobilized polymyxin B were added to the
assay medium. The reaction was initiated by the addition of the sub-
cellular fraction. A and C contain results obtained with palmitoyl-CoA,
whereas B and D contain results obtained with palmitoyl-CoA-agarose.
The mitochondrial and microsomal GAT activities in the absence of
polymyxin B were 2–3 and 3–4 nmol/min/mg, respectively. The values
in the figure are the average of two separate sets of experiments.

FIG. 3. Acylation products formed in the presence of palmitoyl-CoA-
agarose. Mitochondria (0.64 mg/ml) and microsomes (1.76 mg/ml) were incubated
in the GAT assay medium in a total vol-
ume of 0.5 ml. The formation of LPA (■) and PA (□) was analyzed by thin layer
chromatography. The values in the figure are the average of four separate sets of
experiments.
**Mitochondrial Phosphatidic Acid Synthesis**

**Fig. 4. Export of PA from mitochondria.** Mitochondria (0.4–0.6 mg/ml) were incubated in GAT assay medium for 10 min and then diluted five times with ice-cold buffer A (0.25 M sucrose, 10 mM Tris, 2 mM EDTA, pH 7.4) and pelleted by centrifugation at 4 °C, 10,000 × g for 15 min. The sediment was re-suspended in buffer B (20 mM Tris, 10% glycerol, 2 mM EDTA, pH 7.4) and divided into 0.5-ml aliquots. Each aliquot contained 1.42 nmol of PA, 0.42 nmol of glycerides (mono and diacyl), and 0.37 mg of mitochondrial protein. Mitochondria were incubated with shaking for 0, 20, and 40 min at 25 °C and then L-FABP was added at indicated concentrations, and the volume was made up with buffer A to 1 ml. After 5 min, the mitochondria were pelleted by centrifugation at 10,000 × g for 10 min. The supernatant was treated with 1-butanol and PA, and glycerides were separated by thin layer chromatography. Results are presented as PA (L), PA (M), or glycerides (Œ) released after 0-min (L), 20-min (M), 20-min (Œ), or 40-min (Œ) incubation of mitochondria prior to L-FABP addition.

**Fig. 5. Release of LPA, PA, and glycerides from mitochondria.** Mitochondria were loaded with PA and resuspended in buffer B (20 mM Tris/HCl buffer, pH 7.4, 10% glycerol, 5 mM CaCl₂) to 3 mg/ml mitochondrial protein. Crude liver cytosol was added (1 mg/ml), and the incubation was continued for 10 min at 25 °C. The mitochondria were spun down, reconstituted in buffer A, and divided into 0.5-ml aliquots. Each aliquot contained 0.38 nmol of LPA, 0.41 nmol of PA, 0.68 nmol of glycerides, and 0.62 mg of mitochondrial protein. 1-FABP was added at indicated concentrations, and the volume was made up with buffer A to 1 ml. After 5 min, the mitochondria were pelleted by centrifugation. The supernatant was extracted with 1-butanol and LPA (●), PA (□), and glycerides (▲) separated by thin layer chromatography. Results are present as the amount released from one aliquot (0.62 mg of mitochondrial protein).

**Fig. 6. Phospholipase A₂ sensitivity of mitochondrial PA.** Mitochondria were loaded with PA, pelleted by centrifugation, re-suspended in buffer B, and divided in 0.5-ml aliquots. Each aliquot contained 1.56 nmol of PA, 0.36 nmol of glycerides, and 0.44 mg of mitochondrial protein. Phospholipase A₂ (5 µg/ml; ▲, ○) or mixture of phospholipase A₂ (5 µg/ml) and L-FABP (35 µM) (●, ◆) were added either immediately (▲, ○), or PA-loaded mitochondria were incubated for 30 min at 25 °C prior to addition of these proteins (◆, ◆). The mitochondria were pelleted and processed as described in the legend to Fig. 5. Results are presented as the amount released from one aliquot (0.44 mg of mitochondrial protein).

**drial and Microsomal Glycerophosphate Acyltransferase**—It is known that polymyxin B stimulates the mitochondrial GAT and markedly inhibits the microsomal enzyme (Refs. 26–28, Fig. 2A). In the presence of polymyxin B-agarose, mitochondria and microsomes showed activation and inhibition in the range of 80–90% of the values obtained in the presence of free polymyxin B (Fig. 2C). The immobilized polymyxin B is cross-linked to 4% beaded agarose through an amino group with a spacer of 1 carbon. It is, therefore, improbable that the antibiotic can penetrate the MOM. Fig. 2 also includes the results using palmitoyl-CoA-agarose and polymyxin B (Fig. 2B) and palmitoyl-CoA-agarose and polymyxin B-agarose (Fig. 2D). Both the free and immobilized polymyxin B stimulated the mitochondrial and inhibited the microsomal GAT. For reason presently unknown, polymyxin B-agarose, at higher concentrations, is less effective in both stimulating and inhibiting mitochondrial and microsomal GAT, respectively (Fig. 2C and D).

**Acylation Products Formed in the Presence of Immobilized Substrate**—Amounts of LPA and PA synthesized in mitochondria and microsomes using palmitoyl-CoA and palmitoyl-CoA-agarose as acyl donor are documented in Fig. 3. The phospholipids were formed in equal quantities in presence of free palmitoyl-CoA in mitochondria (15). In microsomes, the amount of LPA was about 30%. A similar profile was seen with the use of palmitoyl-CoA-agarose. In the absence of bovine serum albumin, mainly PA was formed in both microsomes and mitochondria.

**Role of L-FABP in the Export of Mitochondrial LPA and PA**—As reported earlier (17), L-FABP stimulates the export of LPA from mitochondria. The presence of L-FABP stimulates LPA synthesis 6-fold but reduced PA synthesis by 50%. In the absence of L-FABP, mitochondria can synthesize significant...
amount of PA, which remains in the mitochondria. However, this PA was marginally exported when mitochondria were exposed to L-FABP (Fig. 4). When mitochondria, loaded with PA, were immediately exposed to 35 μM L-FABP, up to 21% of PA left the mitochondria. The amount of PA available for export decreased with time, suggesting that the PA became inaccessible for binding to L-FABP.

Preferential export of LPA from mitochondria due to L-FABP is documented in Fig. 5. Mitochondria, loaded with PA, were treated with liver cytosol, which resulted in partial conversion of PA to LPA and glycerides. Subsequent exposure of these mitochondria to L-FABP resulted in 94% release of LPA but only 22% release of PA and 6% release of glycerides. Data shown in Fig. 4 suggest that, with time, PA within mitochondria is becoming less available for export. This observation was confirmed by another approach. Mitochondria, loaded with PA, were exposed either to phospholipase A2 alone or to a mixture of phospholipase A2 and L-FABP. LPA, generated by phospholipase A2 from PA, was exported 2.5-fold more efficiently in the presence of either free or immobilized polymyxin B (Fig. 2), it is suggestive that the catalytic site of mitochondrial GAT is exposed to the cytosolic side of the MOM.

If LPA is formed on the outer surface of the MOM, which side of the membrane is PA formed? Analysis of the acylation products (Fig. 3) revealed that both LPA and PA were formed in the presence of the free or agarose-bound palmitoyl-CoA. Absence of bovine serum albumin in the incubation medium similarly affected the LPA:PA ratio with the two forms of the acyl-CoA. As expected (15), in the absence of bovine serum albumin, PA was the main reaction product. These results strongly suggest that both LPA and PA are made on the outer side of the MOM. Formation of both the phospholipids is stimulated in the presence of free or agarose-bound polymyxin B (results not shown), further confirming the site of formation of the phospholipids in the transverse plane of the MOM.

The results obtained here, together with those reported earlier on the formation acyl-CoA on the cytosolic side of the MOM (24), indicate that the three enzymes, acyl-CoA synthetase, GAT, and MGAT, all can draw on the cytosolic pool of substrates. This situation raises the possibility that these three enzymes are closely located and that there could be an efficient substrate “channeling” between these enzymes.

The PA synthesized on the outer surface of the MOM leaves the organelle to a very limited extent (Figs. 4 and 6). It can be converted to diacylglycerol by phosphatidate phosphohydrolase (30) or can be converted back to LPA under certain conditions by phospholipase A2, which is also located in the MOM (31, 32). The fate of the mitochondrially synthesized PA is its conversion to cardiolipin, the final step of which takes place in the inner membrane (33, 34). There is precursor-product relationship between PA and cardiolipin when PA-loaded mitochondria are incubated in a cardiolipin-synthesizing medium. Furthermore, the final step of cardiolipin synthesis takes place on the inner side of the inner membrane (29). Thus, there is a complex topological movement of PA from the outer surface of the outer membrane to the inner surface of the inner membrane. Transport of PA from the outer to inner membrane may occur either by simple diffusion (35) or by some other mechanism, for example, involving some transport protein. However, the movement of PA from the outer to inner membrane fits in with our observation that incubation of PA-loaded mitochondria renders the phospholipid inaccessible to externally added phospholipase A2 (Fig. 6).

It appears that each of the products of the three enzymes,
acyl-CoA synthetase, GAT, and MGAT, has at least two possible fates (Fig. 7). The acyl-CoA synthesized in the mitochondria can either be transported to the mitochondrial matrix for β-oxidation or be acted upon by GAT to form LPA. It is known that fatty acid biosynthesis is regulated by metabolic modulators (36). Similarly, as GAT is influenced by ATP and citrate (22), they can very well be involved in this regulation. Similar to acyl-CoA, the LPA formed in the MOM has also two fates. It can either be acylated to form PA (6), or it can combine with L-FABP and be exported to the endoplasmic reticulum for conversion to PA (17, 37) and presumably to other phospholipids. LPA-FABP does not act as a substrate for mitochondrial MGAT (17). A small amount of PA can leave the mitochondria. Whether this PA is transported to the endoplasmic reticulum for conversion to complex phospholipids is unknown. However, the majority of mitochondrially synthesized PA appears to be converted to cardiolipin. The dual fate of acyl-CoA, LPA, and PA suggest the existence of multiple control sites in the metabolism of these compounds.

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REFERENCES

1. Kennedy, E. P. (1961) Fed. Proc. 20, 934–940
2. Kornberg, A., and Pricer, W. E., Jr. (1953) J. Biol. Chem. 204, 345–357
3. Kennedy, E. P. (1953) J. Biol. Chem. 201, 399–412
4. Monroy, G., Rola, F. H., and Pullman, M. E. (1972) J. Biol. Chem. 247, 6884–6894
5. Daas, L. N. W. (1973) Biochim. Biophys. Acta 306, 186–193
6. Haldar, D., Kelker, C. H., and Pullman, M. E. (1983) Trans. N. Y. Acad. Sci. 41, 173–182
7. Bell, R. M., and Coleman, R. A. (1980) Annu. Rev. Biochem. 49, 459–487
8. Haldar, D., and Vancura, A. (1992) Methods Enzymol. 209, 64–72
9. Monroy, G., Kelker, H. C., and Pullman, M. E. (1973) J. Biol. Chem. 248, 2845–2852
10. Vancura, A., and Haldar, D. (1994) J. Biol. Chem. 269, 27209–27215
11. Yet, S. F., Moon, Y. K., and Sul, H. S. (1995) Biochemistry 34, 7303–7310
12. Nikonov, A. V., Morimoto, T., and Haldar, D. (1996) in Recent Research Development in Lipids Research (Pandalai, S. G., ed), Vol. 2, Part II, pp. 207–222. Transworld Research Network, Trivandrum, India
13. Salem, L. (1962) J. Chem. Phys. 37, 2100–2113
14. van Deenen, L. L. M. (1965) in Progress in Chemistry of Fats and Other Lipids (Holman, R. T., ed) Vol. VIII, Part I, pp. 1–115. Pergamon Press, Oxford
15. Haldar, D., Tso, W., and Pullman, M. E. (1979) J. Biol. Chem. 254, 4502–4509
16. Stern, W., and Pullman, M. E. (1978) J. Biol. Chem. 253, 8047–8055
17. Vancura, A., and Haldar, D. (1992) J. Biol. Chem. 267, 14353–14359
18. Bates, E. J., and Saggerson, E. D. (1979) Biochem. J. 182, 751–762
19. Saggerson, E. D., and Carpenter, C. A. (1987) Biochem. J. 243, 289–292
20. Sul, H. S., and Wang, D. (1998) Annu. Rev. Nutr. 18, 331–351
21. Dricks, L. K., and Sul, H. S. (1997) Biochim. Biophys. Acta 1348, 17–26
22. Nikonov, A. V., and Haldar, D. (1996) FASEB J. 10, A1110
23. Hesler, C. B., Carroll, M. A., and Haldar, D. (1985) J. Biol. Chem. 260, 7452–7456
24. Hesler, C. B., Olymbios, C., and Haldar, D. (1990) J. Biol. Chem. 265, 6600–6605
25. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
26. Carroll, M. A., Morris, P., Grosjean, C. D., Anzalone, T., and Haldar, D. (1982) Arch. Biochem. Biophys. 214, 17–25
27. Das, S. K., and Haldar, D. (1987) Lipids 22, 757–759
28. Fitzpatrick, S. M., Sorresso, G., and Haldar, D. (1982) J. Neurochem. 39, 256–269
29. Schlame, M., and Haldar, D. (1993) J. Biol. Chem. 268, 74–79
30. Smith, M. E., Sedgwick, B., Brindley, D. N., and Hubscher, G. (1967) Eur. J. Biochem. 3, 70–77
31. Nachbaurn, J., and Vignais, P. M. (1986) Biochim. Biophys. Res. Commun. 33, 315–320
32. Schlame, M., and Ruster, B. (1990) Biochem. J. 272, 589–595
33. Hostetler, K. Y. (1982) in Phospholipids (Hawthorne, J. N., and Ansell, G. B., eds) pp. 481–492, Elsevier Science Publishers B. V., Amsterdam
34. Daum, G. (1985) Biochim. Biophys. Acta 822, 1–42
35. Wojtkiewicz, L., Baranska, J., and Zborowski, J. (1990) Biochim. Biophys. Acta 1044, 284–287
36. Sokol, S. J., Stoops, J. K., and Joshi, C. V. (1995) Biochim. Biophys. Acta 1198, 537–579
37. Haldar, D., and Lipfert, L. (1990) J. Biol. Chem. 265, 11014–11016