Transmembrane Movement of Phosphatidylcholine in Mitochondrial Outer Membrane Vesicles*

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One of the steps in the import of phosphatidylcholine (PC) into mitochondria is transmembrane movement across the outer membrane. This process was investigated in vitro using isolated mitochondrial outer membrane vesicles (OMV) from rat liver. 13C-Labeled PC was introduced into the OMV from small unilamellar vesicles by a PC-specific transfer protein (PCTP). The membrane topology of the newly introduced PC was determined from its accessibility to phospholipase A2. Under conditions where the OMV stay intact, externally added phospholipase A2 is able to hydrolyze up to 50% of both the introduced 13C-labeled PC and the endogenous PC. Pool size calculations showed that close to 100% of the PC in the OMV can be exchanged by PCTP. A back-exchange experiment revealed that the introduction of the labeled PC is irreversible. The results demonstrate that newly introduced PC molecules readily equilibrate over both leaflets of the OMV membrane. The kinetics of the PCTP-mediated exchange process indicate that the t1/2 of the transmembrane movement at 30°C is 2 min or less.

Phosphatidylcholine (PC) is the major constituent phospholipid in both the inner and the outer membrane of mammalian mitochondria. The terminal steps of the de novo synthesis of PC (and most other phospholipids) occur primarily on the cytoplasmic surface of the endoplasmic reticulum. Consequently, the biogenesis of mitochondria requires efficient import of PC from the ER. Several mechanisms for the intracellular transport of phospholipids have been proposed (for a recent review see Ref. 3). There is growing evidence that the import of phosphatidylserine into mitochondria occurs via direct interorganellar contact between mitochondria and the so-called mitochondria-associated membrane (4–7). This ER-like membrane contains several phospholipid biosynthetic enzymes including some involved in the synthesis of PC (7, 8). The mechanism of PC import into mitochondria is not known.

Additional mechanisms, so far unknown, are required for the sorting of the newly imported phospholipids within the mitochondrion, in order to maintain the specific phospholipid composition and translaterolipid distribution of both mitochondrial membranes. In vivo labeling studies in hepatocytes employing 14C-labeled choline have shown that radiolabeled PC appears rapidly in the mitochondrial outer membrane, while transfer to the inner membrane is slow (9). This suggests that newly synthesized PC is transferred from the ER to the inner membrane via the outer membrane. In isolated mitochondria, movement of labeled endogenous PC from the outer to the inner membrane has been demonstrated (10). Thus, the outer membrane is the first barrier to be taken by a newly imported PC molecule on its way to the inner membrane or to the inner leaflet of the outer membrane.

In the present study the process of PC movement across the outer mitochondrial membrane is investigated in an in vitro system consisting of mitochondrial outer membrane vesicles (OMV) isolated from rat liver mitochondria. These OMV are sealed and have a right-side-out topology with an almost symmetric distribution of PC over the inner and outer membrane leaflet (11). Phosphatidylcholine-specific transfer protein (PCTP) was used to introduce radiolabeled PC into the OMV from donor vesicles. The use of PCTP also allowed the determination of the pool size of exchangeable PC in the OMV. The transmembrane distribution of the introduced PC was assessed from its accessibility to externally added phospholipase A2 (PLA2). It is concluded that newly introduced 13C-dioleoylphosphatidylcholine ([14C]DOPC) equilibrates rapidly over the OMV membrane with a t1/2 at 30°C of at most 2 min.

**EXPERIMENTAL PROCEDURES**

**Materials**—The radiochemicals 1,2-di-(1-14C)-oleyl-sn-glycero-3-phosphocholine ([14C]DOPC, 114 Ci/mmol), and (1α,2α-3H)cholesteryl oleoyl ether (1H[3H]CE, 37 Ci/mmol) were obtained from New England Nuclear (Brussels, Belgium), and Amersham (Amersham, United Kingdom), respectively. DOPC and dioleoylphosphatidic acid (DOPA) were purchased from Avanti (Birmingham, AL). PCTP was purified from bovine liver and stored at −20°C at a concentration of 0.5 mg/ml in 20 mM Tris-HCl, pH 7.2, 100 mM NaCl, containing 50% glycerol (12). Porcine pancreatic phospholipase A2 (plPLA2) was purified as in Ref. 13. Bee venom phospholipase A2 (bvPLA2), bovine serum albumin (essentially fatty acid-free), trypsin, and soybean trypsin inhibitor were obtained from Sigma. All other chemicals were analytical grade.

Isolation of Mitochondrial OMV—Mitochondria were isolated from the livers of male Wistar rats by a combination of differential centrifugation and isotonic Percoll gradient centrifugation as described (14). OMV were obtained using a modified version of the method recently introduced for mitochondria from Neaurospora crassa (15).

Briefly, the mitochondria were suspended in hypotonic buffer (5 mM K+, pH 7.2, 5 mM EDTA) at a protein concentration of 5 mg/ml and incubated on ice for 20 min under stirring. The outer membrane was detached from the remaining mitoplasts by 20 strokes in a Potter-Elvejem homogenizer with a tightly fitting Teflon pestle. The homogenate (15 ml) was layered on a sucrose step gradient consisting of 9 ml of 0.25 M sucrose and 12 ml of 1.1 M sucrose in 10 mM MOPS, pH 7.2, 2.5.

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1 The abbreviations used are: PC, phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DOPA, dioleoylphosphatidic acid; CE, cholesteryl oleoyl ether; MOPS, 3-(N-morpholino)propanesulfonic acid; PLA2, phospholipase A2; ppPLA2, porcine pancreatic PLA2; bvPLA2, bee venom PLA2; OMV, outer membrane vesicles; PCTP, PC-specific transfer protein; SUV, small unilamellar vesicles; ER, endoplasmic reticulum; HPTLC, high performance thin layer chromatography.
was counted, and the percentage of "$^{14}$C"DOPC extracted from the OMV was calculated, taking into account the above corrections, while assuming that 10% of the SUV with associated "$^{14}$C" label co-pellets with the OMV.

Phospholipase A$_2$, Treatment—OMV at a protein concentration of 0.6 mg/ml in MSH buffer were incubated for the indicated periods with ppPLA$_2$, and bvPLA$_2$, at a concentration of 0.4 units/ml each, in the presence of 0.1 mM Ca$^{2+}$ at room temperature. This mixture of phospholipases A$_2$ is optimal for determining the phospholipid topology in OMV, as stable levels of phospholipid degradation are obtained rapidly (11). PLA$_2$ activity was inhibited by the addition of 0.75 mM ETA. The OMV were pelleted (20 min at 185,000 $\times$ g) and subjected to phospholipid extraction. The specific activities of ppPLA$_2$ (1.25 $\times$ 10$^3$ units/mg) and bvPLA$_2$ (4.2 $\times$ 10$^3$ units/mg) were determined using egg yolk lipoproteins as substrate (13).

Intactness of the OMV—After treatment with PLA$_2$, aliquots of the OMV suspension corresponding to 40% of protein were incubated with 1.7 mg of trypsin/mg of OMV for 20 min on ice, prior to the addition of a 2.5-fold excess (w/w) of soybean trypsin inhibitor over trypsin (11). The remaining activity of the enzyme adenylate kinase, determined spectrophotometrically at 37°C (14, 21), served as a measure for the intactness of the OMV.

Analysis of PC Degradation—Phospholipids were extracted according to the method of Rose and Oklander (22) from samples corresponding to 150 mg of OMV. Intactness of the OMV was assessed by two-dimensional HPTLC on silica gel 60 (Merck, FRG), using chloroform, methanol, 25% ammonia, water (90:5:4:5:5:5:5 (v/v/v/v)).

As the hydrolysis of "$^{14}$C"DOPC to lyso-"$^{14}$C"PC and "$^{14}$C"oleate by PLA$_2$ did not affect the recovery of the radiolabel from the extraction procedure (see Fig. 1A), it was possible to quantitate the extents of "$^{14}$C"DOPC degradation from the HPTLC elution profiles. No hydrolysis of PC was detected in control experiments without PLA$_2$.

Other modifications—Protein concentrations were determined by the BCA assay (Pierce) with 0.1% (w/v) SDS added and bovine serum albumin as standard. Concentrations of SUV were determined by phosphate analysis (17).

RESULTS

Localization of "$^{14}$C"DOPC in OMV by Treatment with PLA$_2$, "$^{14}$C"-Labeled DOPC was introduced into isolated mitochondrial OMV from donor vesicles by the action of the PCTP. This protein catalyzes a one-to-one PC exchange resulting in a replacement of the endogenous PC in the acceptor membrane with PC from the donor vesicles, i.e., without changing the PC content of the membrane (22, 25). "$^{14}$C"DOPC was efficiently transferred from donor SUV to acceptor OMV under the conditions used, an incubation for 20 min at 30°C with equal amounts of PC present in the populations of donor and acceptor vesicles (PC$_{donor}$/PC$_{acceptor}$ molar ratio approximately 1), and in the presence of PCTP. Typically, around 4 nmol of "$^{14}$C"DOPC were transferred per mg of OMV protein.

After removal of the donor SUV by centrifugation, the OMV were incubated with a mixture of phospholipases A$_2$, from bee venom and porcine pancreas to determine the membrane topology of the labeled PC molecules. Fig. 1A shows the HPTLC analysis of OMV phospholipid extracts from a typical experiment. Approximately 40% of the "$^{14}$C"DOPC is hydrolyzed within 10 min by the action of PLA$_2$ (Fig. 1, A and B). Degradation of the remainder proceeds slowly and is accompanied by a gradual loss of the intactness of the OMV as indicated by the increasing accessibility of adenylate kinase present in the OMV lumen to externally added trypsin (Fig. 1C). In the presence of an excess of PLA$_2$ (Fig. 1A), up to 94% of the "$^{14}$C"DOPC is degraded in 10 min. This is accompanied by a loss of barrier function of the OMV membrane as evidenced by the proteolysis of over 80% of adenylate kinase by trypsin (data not shown).

Under similar conditions with an excess of PLA$_2$, present virtually all endogenous PC is hydrolyzed (11).
The time course of the degradation of $^{14}$C]DOPC parallels that of the hydrolysis of endogenous PC under the same conditions (Fig. 1B). From the amount of $[^3H]$CE present in the lipid extracts (Fig. 1A) it is estimated that approximately 10% of the total $^{13}$C label present originates from co-pelleted SUV. This contamination may account for the slightly different degrees of protection from PLA$_2$ observed for labeled and endogenous PC after 10 min of incubation (Fig. 1B). When the time of the incubation with PCTP is reduced to 3 min, still 60 $\pm$ 5% of the $^{14}$C label is recovered as intact PC after 10 min of treatment with PLA$_2$ (data not shown). The data suggest that the newly introduced PC rapidly adopts a transbilayer distribution similar to that of the endogenous PC, with approximately 50% of the PC content localized in the inner leaflet and 50% in the outer leaflet in agreement with Ref. 11.

The Exchangeable Pool of PC in the OMV—In order to calculate the specific radioactivity of the PC pool in the OMV after exchange by PCTP, the PC content of the OMV was determined. The OMV have a phospholipid phosphorus/protein ratio of 1220 $\pm$ 150 nmol/mg (S.D., $n = 6$) and a PC content of 54.7 $\pm$ 3.1% (S.D., $n = 11$) (compare Refs. 1 and 14).

The existence of a fast equilibration of newly introduced $^{14}$C]DOPC across the OMV membrane is supported by the results depicted in Fig. 2. The specific radioactivity of the PC pool in the OMV was determined after different times of exchange by PCTP and compared with that of the outer leaflet of the donor SUV, as PCTP only has access to the outer leaflet of phospholipid vesicles (19). After 5 min of exchange, the specific radioactivity of both PC pools reaches a similar equilibrium value (Fig. 2), demonstrating that the entire PC pool of the OMV has exchanged with the outer leaflet of the SUV. The exchangeable pool of PC in the OMV was determined in several independent experiments, yielding an average value of 91 $\pm$ 12% (S.D., $n = 22$).

After introduction into the OMV membrane, $^{14}$C]DOPC is completely extractable in a back-exchange experiment as shown in Fig. 3. OMV with $^{14}$C]DOPC incorporated were incubated with an excess of unlabeled SUV (PC SUV/PCOMV molar ratio of 10) and PCTP. Under these conditions all of the $^{14}$C]DOPC is in exchange with the PC in the outer leaflet of the SUV (Fig. 3), demonstrating the reversibility of the transmembrane movement.

**DISCUSSION**

The present study demonstrates that $^{14}$C]DOPC introduced into the outer leaflet of isolated mitochondrial outer membrane vesicles by PCTP is rapidly equilibrated over the two membrane leaflets. This conclusion is based on two lines of experimental evidence. (i) Similar to the extent of hydrolysis of endogenous PC, only 50% of the introduced $^{14}$C]DOPC can be degraded by externally added PLA$_2$, while the OMV stay intact, indicating that the other 50% has flipped to the inner leaflet. (ii) The pool size of exchangeable PC in the OMV is close to 100%. This result, combined with the reversibility of the exchange process (Fig. 3), indicates that the probe molecule $^{14}$C]DOPC behaves like endogenous PC. The monophasic kinetics observed for the PCTP-catalyzed PC exchange process (Fig. 2) do not allow distinction between the rates of the actual exchange and the PC transmembrane movement. Consequently, the rate constant of the transmembrane movement of PC (flip-flop) is close to or higher than the rate constant of the overall exchange process, which implies that at 30°C the $t_{1/2}$ for PC transmembrane movement in OMV is 2 min or less.

![Figure 1](image-url)
Phosphatidylcholine exchange by PCTP between OMV and \([^{14}C]DOPC\)-labeled SUV results at equilibrium in similar specific radioactivity values for the PC pool of the OMV and the PC present in the outer leaflet of the SUV. OMV at a concentration of 0.25 mg/ml were incubated with PCTP (18 \(\mu\)g/ml) and radiolabeled SUV (0.15 mM PC) at 30°C. At the indicated time points OMV and SUV were separated by centrifugation, and the radioactivity in pellet and supernatant was determined. The specific radioactivity of PC in the OMV (●) and in the outer leaflet of the SUV (△) was calculated as described under "Experimental Procedures."

Direct access of PCTP to the inner leaflet of the OMV membrane by permeation could in principle also explain the results obtained in the present study. However, this possibility is considered remote in view of the molecular size of PCTP (25 kDa (26)) relative to that of cytochrome c (12 kDa), for which the outer membrane is impermeable (27). Accordingly, labeled PC is not recovered in the inner membrane fraction after its introduction into intact mitochondria by PCTP (20).

It may seem paradoxical that on the one hand almost 100% of the PC pool of the OMV is accessible to PCTP, while on the other hand only half of the introduced \([^{14}C]DOPC\) can be degraded by external PLA2 in intact OMV (Fig. 1). A possible explanation would be that the PCTP-catalyzed exchange process, or PCTP itself, induces the rapid transmembrane movement of PC in OMV. This possibility cannot be excluded; however, so far there are no indications that PCTP would have this ability in other biomembranes (23, 24, 28). Furthermore, PCTP does not induce phospholipid transbilayer movement in protein-free phospholipid vesicles (17, 18). The alternative explanation, i.e. that the lyso-PC molecules produced by PLA2 do not participate in the transmembrane equilibration of PC, is considered more likely.

The microsomal contamination present in the preparations of OMV requires consideration in view of the rapid rate of PC flip-flop occurring in rat liver microsomes (8 min \(\sim\) 2–3 min at 37°C (17)) and the proposed role of a specialized ER fraction (mitochondria-associated membrane) in phospholipid transport to mitochondria (4, 8). Based on the specific activity of the microsomal marker enzyme glucose-6-phosphatase in OMV preparations and in microsomes isolated according to Ref. 14, the microsomal contamination of the OMV does not exceed 9.4% based on protein. Given the phospholipid:protein ratio of microsomes (1), it can be calculated that this percentage corresponds to a contamination of at most 4% on a phospholipid basis. Consequently, microsomal PC hardly contributes to the total PC pool. Vance (8) has shown that the specific activity of glucose-6-phosphatase is higher in mitochondria-associated membrane than in microsomes, whereas that of NADPH-cytochrome c reductase is lower. There are no indications that the microsomal fraction contaminating the OMV has characteristics of mitochondria-associated membrane, as the specific activities of both enzymes in the OMV relative to those in the rat liver homogenate are similar (0.45 and 0.55, respectively).2

In vitro studies on the import of PC by rat liver mitochondria have shown that labeled PC is confined to the outer membrane after its introduction by PCTP from phospholipid vesicles (11, 20). These results raised the question of which of the putative consecutive steps in intramitochondrial PC transport is limiting, the movement across the outer membrane or the transfer from outer to inner membrane. The present study strongly argues that the first step is not limiting. Calculations of the pool size of exchangeable PC in the outer membrane of intact mitochondria (20) suggest that the occurrence of transmembrane equilibration in the outer membrane depends on the acyl chain composition and the type of label of the PC molecule. In the case of \([^{14}C]DOPC\) the size of the exchangeable PC pool in the outer membrane of intact mitochondria was found to be 56%, indicating that the label stays in the outer leaflet (11). Combined with the present results this suggests that the inner membrane might have a regulatory role in the transmembrane movement of PC across the outer membrane.

In a study on the topology of phospholipids in mitochondrial OMV from Saccharomyces cerevisiae, the transmembrane movement of PC in the outer membrane was reported to proceed relatively slowly (8 min \(\sim 50\) min (29)). These authors used a different, non-PC-specific transfer protein and a different donor-acceptor system, which complicates direct comparison with the present data.

In vivo labeling in rat hepatocytes has shown that 15 min after the addition of \([^{14}C]choline\), the specific radioactivity in the mitochondrial outer membrane is approaching equilibrium with that in the microsomes, which is consistent with a rapid.

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**Fig. 2.** Phosphatidylcholine exchange by PCTP between OMV and \([^{14}C]DOPC\)-labeled SUV results at equilibrium in similar specific radioactivity values for the PC pool of the OMV and the PC present in the outer leaflet of the SUV. OMV at a concentration of 0.25 mg/ml were incubated with PCTP (18 \(\mu\)g/ml) and radiolabeled SUV (0.15 mM PC) at 30°C. At the indicated time points OMV and SUV were separated by centrifugation, and the radioactivity in pellet and supernatant was determined. The specific radioactivity of PC in the OMV (●) and in the outer leaflet of the SUV (△) was calculated as described under "Experimental Procedures."

**Fig. 3.** The transfer by PCTP of \([^{14}C]DOPC\) from donor vesicles to OMV is reversible. Shown is the back-exchange experiment, in which \([^{14}C]DOPC\)-labeled OMV (0.25 mg protein/ml) were incubated with an excess of unlabeled SUV (1.5 mM PC) and PCTP (18 \(\mu\)g/ml) at 30°C for different times. After centrifugation, the radioactivity in the pellet (OMV) and the supernatant (SUV) was determined, and the amount of \([^{14}C]DOPC\) transferred from the OMV was calculated. The data are presented as percentage of the maximum amount of extractable \([^{14}C]DOPC\) expected at equilibrium, i.e. when the specific radioactivities of OMV and the outer leaflet of the SUV have reached the same value.
transmembrane movement of PC in the outer membrane (9).

The existence of rapid PC transmembrane movement across the mitochondrial outer membrane makes a lot of sense from the point of view of biology. Since mitochondria are highly dynamic organelles that should have easy access to newly synthesized membrane components, it does not come as a surprise that the outer membrane has a mechanism for rapid shuttling of phospholipids. Comparable fast rates of energy-independent phospholipid transmembrane movement have been reported in membranes that are sites of phospholipid synthesis, the ER membrane (18, 30) and the inner membrane of Escherichia coli (31).

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