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Transbilayer movement of phosphatidylcholine in the mitochondrial outer membrane of *Saccharomyces cerevisiae* is rapid and bidirectional

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Abstract

The process of transmembrane movement of phosphatidylcholine (PC) across the outer membrane of mitochondria was investigated in vitro in mitochondrial outer membrane vesicles (OMV) from the yeast *Saccharomyces cerevisiae*. Phosphatidylcholine-transfer protein (PC-TP) was used to extract radiolabeled PC from OMV, with small unilamellar vesicles serving as acceptor system. Endogenously radiolabeled PC synthesized either via the CDP-choline pathway or via methylation of phosphatidylethanolamine can be extracted completely from the OMV with a $t_{1/2}$ of 1 min or less at 30°C. The size of the pool of PC in OMV available for exchange by PC-TP is not affected by pretreatment of the OMV with proteinase K or sulfhydryl reagents. In the reverse experiment where radiolabeled PC was introduced into the OMV, similar characteristics for the exchange were found. The accessibility of labeled PC to externally added phospholipase A_2 was used as a measure for its transmembrane distribution. It was found that PC is not exclusively located in the outer leaflet of the OMV. Only 30–35% can be degraded in intact OMV by phospholipase A_2, irrespective of whether the PC is introduced by PC-TP or endogenously synthesized via either of the pathways of biosynthesis. The results demonstrate the occurrence of rapid bidirectional transbilayer movement of both endogenous and in vitro introduced PC in OMV. Furthermore, there appears to be no preference for mitochondrial import of PC synthesized by either of the pathways in vivo.

Keywords: Flip-flop; Phosphatidylcholine; Mitochondria; Yeast; Outer membrane; Phospholipid transport

1. Introduction

Phosphatidylcholine (PC) is the major phospholipid found in membranes of eukaryotic cells. As in higher eukaryotes, PC is synthesized via two distinct pathways in yeast, either via the triple methylation of PE or via the CDP-choline (Kennedy) pathway (for a recent review, see [1]). In yeast, the methylation of

Abbreviations: (bv)PLA2, (bee venom) phospholipase A2; CE, cholesteryl oleoyl ether/ester; (DO)PC, (dioleolyl)phosphatidylcholine; ECL, enhanced chemiluminescence; EGTA, ethylene glycol-bis(ß-amino-ethyl ether)*N,N,N′,N′*-tetraacetic acid; FITC, fluorescein isothiocyanate; (HP-)TLC, (high-performance) thin-layer chromatography; MAM, mitochondria-associated membrane; MOPS, 3-(*N*-morpholino)propane-sulfonic acid; NEM, *N*-ethylmaleimide; OMV, outer membrane vesicles; PAGE, polyacrylamide gel electrophoresis; PCMBS, *para*-chloromercuribenzene sulfonic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; SDS, sodium dodecyl sulfate; SRA, specific radioactivity; SUV, small unilamellar vesicles; OG, octylglucoside; PC-TP, PC-specific transfer protein

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PE is considered to be the primary pathway of biosynthesis of PC when cells are grown in the absence of choline, whereas the CDP-choline pathway is an auxiliary route since it requires exogenous choline for net PC synthesis [2].

PC is also a major constituent phospholipid of both mitochondrial membranes [3]. Several mutant strains with defects in the routes of biosynthesis of PC have the tendency to generate respiratory-deficient petites at high frequency [4], suggesting that PC is required for proper mitochondrial function or biogenesis and maintenance. Mitochondria do not contain enzymatic activities capable of PC biosynthesis, the contribution of mitochondria to cellular phospholipid biosynthesis is restricted to the formation of PE and cardiolipin [5]. The final steps of both routes of PC biosynthesis take place in the endoplasmic reticulum. For the biogenesis of mitochondria, PC must be imported efficiently from its site of synthesis. The mechanisms and regulatory aspects involved in the mitochondrial import and sorting of PC over both mitochondrial membranes, as well as the relative contributions of the two biosynthetic pathways to mitochondrial PC, are unknown. Several mechanisms for the intracellular transport of phospholipids have been proposed [6]. A combination of intermembrane and transmembrane transport steps is expected to be required for import of PC into mitochondria and the intramitochondrial transport of PC. Previous studies on PC import into yeast mitochondria showed that PC was able to reach the inner membrane when the outer leaflet of the outer membrane was loaded with PC in vitro. This intramitochondrial transfer of PC required neither an electrochemical gradient across the mitochondrial inner membrane nor ATP [7]. Furthermore, in vivo pulse-labeling studies showed that PC transfer between endoplasmic reticulum and mitochondria continued in the absence of metabolic energy, although at a lower rate as compared to energy-supplemented cells [8].

The mitochondrial outer membrane, which is the site of interaction with the cytosol and other organelles, appears to be the first barrier to be taken by newly synthesized phospholipids on their way into the mitochondrion. In this study the transmembrane movement of PC across the mitochondrial outer membrane was investigated in an in vitro approach using isolated mitochondrial outer membrane vesicles (OMV) from the yeast *Saccharomyces cerevisiae*. A phosphatidylcholine specific transfer protein (PC-TP) was used as a tool to extract radiolabeled PC from the OMV or to introduce radiolabeled PC into the OMV, which allowed the determination of the pool size of PC in the OMV which is available for exchange. The accessibility to externally added phospholipase A_2 was assessed to compare the transmembrane distribution of newly introduced and endogenous PC. The results obtained demonstrate the occurrence of rapid transmembrane movement of both endogenous and in vitro introduced phosphatidylcholine in isolated mitochondrial outer membrane vesicles with a $t_{1/2}$ of 1 min or less at 30°C. The newly introduced radiolabeled PC adopts a transmembrane distribution similar to that of endogenous PC synthesized via either of the two biosynthetic pathways. In vivo labeling demonstrated no apparent preference of the mitochondria for PC synthesized by one of the routes of biosynthesis.

2. Materials and methods

2.1. Materials

The radiochemicals [1α,2α(n)-^{3}H]cholesteryl oleoyl ether ([^{3}H]CE, 48 Ci/mmol), cholesteryl [1-^{14}C]oleoyl ester ([^{14}C]CE, 56 mCi/mmol), L-[methyl-^{3}H]methionine ([^{3}H]methionine, 79 Ci/mmol), and L-[3-^{3}H]-serine were obtained from Amersham (Amersham, UK), 1,2-di-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine ([^{14}C]DOPC, 114 Ci/mol) and [methyl-^{14}C]-choline chloride ([^{14}C]choline, 54 Ci/mol) were purchased from DuPont NEN (Brussels, Belgium). DOPC and dioleoylphosphatidic acid (DOPA) were obtained from Avanti Polar Lipids (Birmingham, AL). PC-TP 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 [9]. Bee venom phospholipase A_2 (bvPLA$_2$), trypsin, proteinase K, FITC–dextran (molecular mass 20 000), octylglucoside, choline oxidase from *Alcaligenes* species, and horseradish peroxidase type II were obtained from Sigma. Enhanced chemiluminescence (ECL) reagents were purchased from DuPont NEN. Zymolyase was obtained from

Seikagaku (Japan). All other chemicals were analytical grade.

2.2. Isolation of outer membrane vesicles and other subcellular fractions

The wild-type yeast strain *S. cerevisiae* D273-10B was grown aerobically to late log (OD_{600} 4–5 (Perkin Elmer Lambda 18 UV/Vis spectrophotometer)) at 30°C in semi-synthetic lactate medium [10]. Spheroplasts were prepared using zymolyase as described previously [10]. The isolation of mitochondria at pH 6.0 and further purification by sucrose gradient centrifugation were based on published procedures [10–12]. Microsomes were isolated as the $32\,500\times g$ pellet of a $20\,200\times g$ post-mitochondrial supernatant. The isolation of mitochondria associated membranes (MAM) was adapted from a published procedure [12]. Mitochondrial outer membranes were isolated and purified based on Mayer et al. [13,14]. Full details of all fractionation procedures are presented elsewhere [15]. The final outer membrane pellet was resuspended in HS buffer (2 mM Hepes (pH 7.4) and 200 mM sucrose). This buffer is used in all experiments unless indicated otherwise. For the preparation of OMV containing radiolabeled phosphatidylcholine synthesized either by the CDP-choline pathway or via methylation of PE, mitochondria were isolated from cells grown in the presence of [^{14}C]choline (125 μCi/l) or [^{3}H]methionine (1500 μCi/l), respectively. The radiolabeled mitochondria were mixed with an approximately 24-fold quantity of unlabeled mitochondria for the isolation of the outer membranes. Approximately 98% of the radiolabel in OMV was present in PC when [^{14}C]choline was used and approximately 90% of the lipid-associated radiolabel in OMV was present in PC when [^{3}H]methionine was used.

2.3. Preparation of small unilamellar vesicles

Small unilamellar vesicles (SUV) were prepared by sonication of a lipid suspension obtained by hydrating a dry lipid film with HS buffer. The lipid film consisted of DOPC and DOPA at a 95:5 molar ratio and the appropriate amounts of radiochemicals. SUV used as donor vesicles for the introduction of [^{14}C]DOPC into OMV contained 0.5 μCi of [^{14}C]DOPC and 5 μCi of [^{3}H]CE per μmol of phospholipid, whereas SUV used as acceptor vesicles in the extraction of endogenously synthesized [^{14}C]PC or [^{3}H]PC only contained 0.5 μCi of [^{3}H]CE or 50 nCi of [^{14}C]CE, respectively, per μmol of phospholipid. The phospholipid suspension was subjected to 10 cycles of 30 s ultrasonication at 80 W and 0°C with 30-s intervals using a Branson B12 sonifier equipped with a microtip. The sonicated phospholipid suspension was centrifuged for 30 min at $390\,000\times g$ and 4°C in a Beckman TL-100 ultracentrifuge. The supernatant containing the SUV was stored at 4°C and used within 2 days after preparation.

2.4. Exchange of PC by PC-TP

OMV (at a protein concentration of 0.25 mg/ml) were incubated with SUV at the indicated ratios and PC-TP (23 μg/ml) at 30°C. The ratio of donor and acceptor membranes is always expressed as the molar ratio of PC in SUV over PC in OMV. After the indicated times the OMV and the SUV were separated by centrifuging for 20 min at $230\,000\times g$ in a Beckman TL-100 ultracentrifuge at 4°C. ^{14}C and ^{3}H were counted in samples from the incubation mixture and from the supernatant using a Packard 1500 Tricarb Liquid Scintillation analyzer. In the case where OMV containing [^{3}H]PC (labeled via the methylation pathway) were used, dried lipid extracts of the samples were counted, because the radiolabel was not exclusively lipid-associated. The extent of the PC exchange was calculated from the enrichment or depletion of the radiolabeled PC in the supernatant containing the SUV. When endogenously radiolabeled OMV were used, a correction was made for the amount of radiolabel associated with lipids other than PC. Furthermore, a correction was made for the contamination of the OMV with co-pelleted SUV using the ^{3}H- or ^{14}C-labeled nonexchangeable marker CE. This contamination typically amounted to 10% of the total amount of SUV added. In the calculation it was assumed that the co-pelleted SUV have specific radioactivities identical to those in the supernatant. In addition to the above-mentioned considerations, the calculation of the pool size of exchangeable PC in the OMV was based on the notion that 65% of the PC in the donor SUV is located

in the outer leaflet and thus accessible to PC-TP [16–18]. The OMV pellets obtained after introduction of [^{14}C]DOPC by PC-TP for 20 min at 30°C were resuspended in HS buffer and kept on ice until treatment with phospholipase A_2.

2.5. Pretreatment of OMV

Where indicated, OMV (at a concentration of 0.19 mg/ml) were incubated with proteinase K (200 μg/ml) for 10 min at 37°C, or with *para*-chloromercuribenzene sulfonic acid (PCMBS, 2 mM), or with *N*-ethylmaleimide (NEM, 5 mM) for 10 min at room temperature after a 10-min preincubation at room temperature with dithiothreitol (DTT, 1 mM). After the various incubations, the suspensions were put on ice, diluted twofold with buffer (to the proteinase K-treated sample 1 mM phenylmethylsulfonyl fluoride was also added) and the OMV were reisolated by centrifugation for 20 min at 230 000 × *g* in a Beckman TL-100 ultracentrifuge at 4°C. The pellets were resuspended in HS buffer and subjected to exchange of PC by PC-TP as above.

2.6. Treatment with phospholipase A_2

OMV (at a protein concentration of 0.6 mg/ml) were incubated for 10 min at room temperature with bvPLA$_2$ at the indicated concentrations in the presence of 0.1 mM Ca^{2+}. The specific activity of the phospholipase ($4.2 \cdot 10^3$ U/mg) was determined using egg yolk lipoproteins as substrate [19]. Octylglucoside (OG, 40 mM) was used in control experiments to solubilize OMV for maximal degradation of phospholipids by bvPLA$_2$. Phospholipase activity was inhibited by the addition of EGTA to a final concentration of 1 mM, and samples were analyzed as described in the following.

2.7. Analysis of PC degradation

Phospholipid extracts from samples corresponding to 40–45 μg of protein were analyzed by HP-TLC on silica gel 60, using chloroform/methanol/25% ammonia/water (90:54:5.5:5.5, v/v/v/v) as eluent. The radioactive spots on the TLC plate were quantified by a Berthold Automatic TLC linear analyzer (Wildbad, Germany). The degradation of PC by bvPLA$_2$ was calculated from the amounts of label present in lysoPC and PC. The recovery of radiolabel was not affected by the extent of hydrolysis of PC. No hydrolysis of the newly introduced [^{14}C]DOPC was detected in control experiments without PLA$_2$. The hydrolysis of PC in OMV containing endogenously synthesized radiolabeled PC by bvPLA$_2$ was calculated after correction for the minor amounts of lysoPC present in these vesicles in untreated controls ($< 4\%$, expressed as percentage of the sum of radiolabeled lysoPC and PC).

2.8. Assessment of the intactness of the OMV

The intactness of the OMV after the phospholipase treatment was assessed by probing the accessibility of Tom40p to trypsin. 2.5-μg aliquots of the OMV suspension were incubated at a protein concentration of 50 μg/ml with trypsin (100 μg/ml) for 20 min on ice. Digitonin (0.2% (w/v)) was added to solubilize the OMV in control experiments. Samples were precipitated with trichloroacetic acid and subjected to SDS–PAGE (10% gel) and Western blotting. To check the intactness during PLA$_2$ treatment, FITC–dextran with a molecular mass of 20 000 was introduced in the lumen of the OMV by a freeze–thaw technique essentially as described for isolated mitochondrial outer membrane vesicles from *Neurospora crassa* [14] with the following changes. The inclusion buffer contained 10 mM MOPS (pH 6.5), 0.5 mM EDTA, 0.5 mM EGTA and 0.5% (w/v) BSA. Snap-freezing was performed in the presence of 0.5 mM FITC–dextran at an OMV concentration of 2 mg/ml. The sucrose step gradient to remove non-enclosed FITC–dextran consisted of the sample, mixed with 50% sucrose and 150 mM KCl, overlaid with 45% sucrose and 150 mM KCl, 45% sucrose and 8% sucrose, respectively, all in 10 mM MOPS (pH 7.2), 2.5 mM EDTA (EM-buffer). After centrifugation for 30 min at 150 000 × *g* in a Beckman SW60 rotor, the OMV were harvested from the 8–45% sucrose interface, diluted with EM-buffer and pelleted by centrifugation. The pellets, containing the OMV with FITC–dextran enclosed in the lumen, were resuspended in H/S buffer and used directly for exchange of PC by PC-TP followed by treatment with bvPLA$_2$ as described. After the inactivation of the phospholipase, samples corresponding to 2.5 μg

OMV were diluted with buffer and the OMV were pelleted by centrifugation. To determine the leakage of the enclosed fluorophore from OMV during phospholipase treatment, the fluorescence in pellet and supernatant was measured in the presence of 0.1% (v/v) Triton X-100. Fluorescence was measured on an Aminco SLM500C spectrofluorometer using an excitation wavelength of 465 nm and an emission wavelength of 543 nm (bandpass 5 nm).

2.9. Other methods

Protein concentrations were measured using the BCA method (Pierce) with 0.1% (w/v) SDS added and bovine serum albumin (BSA) as a standard. Phospholipids were extracted according to the method of Bligh and Dyer [20]. The phosphorus content of the organic phase obtained after extraction was determined to yield the phospholipid phosphorus/protein ratio. Phosphate was determined by the method of Fiske and Subbarow [21]. To determine the PC content of the OMV, phospholipid analysis of lipid extracts from 200 μg OMV was performed by TLC as described [22]. The choline content of the yeast extract used to prepare the culture medium was measured using an enzymatic assay based on the specific oxidation of choline by choline oxidase [23]. Samples were vortexed with finely ground charcoal to remove interfering substances and an internal standard was used to correct for any remaining interference. In Western blotting experiments, protein bands of interest were visualized by ECL. When necessary, quantification of protein bands was performed using laser scanning densitometry on an Ultroscan XL (Pharmacia-LKB, Bronna, Sweden). PS synthase activity (expressed as nmol serine metabolized per min per mg protein) in several subcellular fractions was determined in the presence of Triton X-100 as described [24], at a concentration of 0.1 mM L-[3-^{3}H]serine.

3. Results

3.1. Characterization of the OMV

In this study, the transmembrane movement of PC in the mitochondrial outer membrane from yeast was investigated in isolated outer membrane vesicles (OMV). The purity of this membrane preparation was assessed by immunoblotting. Fig. 1 shows that compared to the purified mitochondria, the OMV are almost completely depleted of the inner membrane marker P_iC (phosphate carrier) and highly enriched in the outer membrane marker Tom20p. Furthermore, the purified mitochondria are depleted of the endoplasmic reticulum marker Sec61p compared to the homogenate and the crude mitochondria (Fig. 1). It was calculated from quantitative blotting experiments (not shown) that the contamination of the OMV with endoplasmic reticulum is at most 5% based on protein. This corresponds to an even lower contamination on a phospholipid basis, as can be inferred from comparison of the phospholipid/protein ratios of OMV and microsomes [3,25]. Therefore, the contribution of microsomal PC to the total PC pool in OMV can be neglected. The OMV have a phospholipid phosphorus/protein ratio of

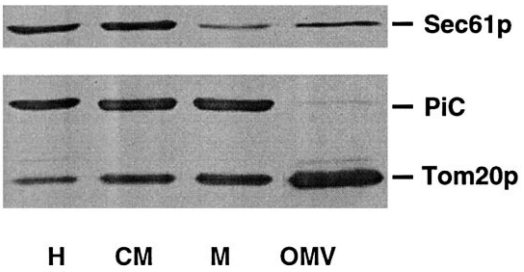

Fig. 1. The purity of OMV assessed by immunoblotting. Samples corresponding to 10 μg protein of the homogenate (H), the crude mitochondria (CM), purified mitochondria (M) and OMV were subjected to SDS–PAGE followed by Western blot analysis using antibodies raised against marker proteins for the endoplasmic reticulum (Sec61p) and the mitochondrial inner and outer membranes (P_iC (phosphate carrier) and Tom20p, respectively). The protein bands were visualized by ECL.

Table 1
Activity of PS synthase in subcellular fractions

Fraction	Relative specific activity
Homogenate	1.0
Crude mitochondria	2.6 ± 0.4
Purified mitochondria	0.5 ± 0.2
Outer membrane vesicles	1.0 ± 0.3
Microsomes	16 ± 4
Mitochondria-associated membranes	35 ± 5

The activity of PS synthase in several subcellular fractions was measured and related to the specific activity of the homogenate.

$14.2 \pm 2.0 \cdot 10^2$ nmol/mg ($n = 7$) and a PC content of $35.8 \pm 2.5\%$ ($n = 4$) of total phospholipid. A specific subfraction of the endoplasmic reticulum which remains associated with the mitochondria upon isolation, the so-called MAM, is highly enriched in PS synthase [12]. Therefore, the activity of this phospholipid biosynthesis enzyme was measured in the OMV and other subcellular fractions. The analysis of PS synthase activity showed that contamination of OMV with the MAM is very low (Table 1) and confirms the minor contamination of OMV with endoplasmic reticulum. The characteristics of the OMV from wild-type yeast strain D273-10B reported here are in agreement with those obtained for strain D273-10B/A1 [15].

3.2. Extraction of endogenously radiolabeled PC from OMV by PC-TP

Exchange experiments using PC-TP provide information on the occurrence of transmembrane movement of PC. PC-TP catalyzes a one-to-one exchange of PC between membranes, which results in the replacement of the endogenous PC in the acceptor membranes with PC from the donor membranes without changing the PC content of the participating membranes [26]. Therefore, the exchange of PC by PC-TP between OMV and small unilamellar vesicles (SUV) was investigated.

For this purpose, OMV containing endogenous radiolabeled PC synthesized via the CDP-choline pathway were isolated from cells grown on medium containing [^{14}C]choline. Because there is no de novo biosynthesis of choline in yeast (choline can only be obtained by uptake from the extracellular medium or by turnover of PC [2]), this also allowed for the assessment of the contribution of choline in the medium to the production of PC via the CDP-choline pathway. If PC were synthesized exclusively via the CDP-choline pathway, the specific radioactivity of cellular PC would be equal to the specific radioactivity of the choline provided in the medium. Therefore, the choline content of the medium was determined, as well as the radioactivity incorporated into the phospholipid. In Table 2 the specific radioactivities of the medium and the whole cell homogenate, mitochondria, and microsomes are compared. The relative contribution of the choline in the medium to cellular PC was found to be approximately 20%. The

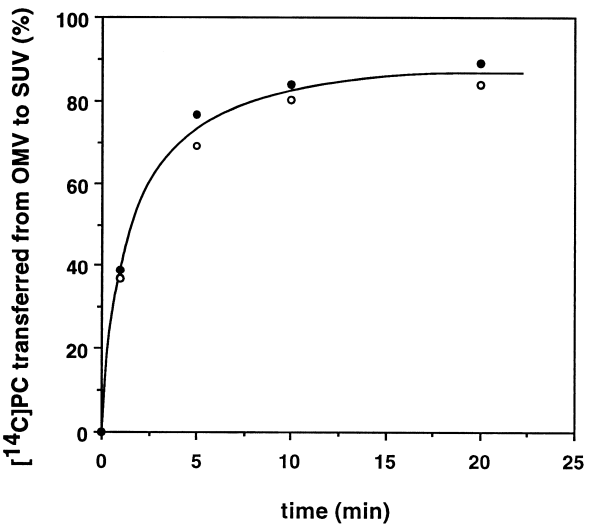

Fig. 2. Time course of the extraction of endogenous PC from OMV by PC-TP. The percentage of ^{14}C-label transferred from the OMV to the SUV, corrected for the contamination of the OMV pellet with SUV, was determined in two independent experiments (●,○). OMV containing endogenous [^{14}C]PC synthesized via the CDP-choline pathway were incubated at a concentration of 0.25 mg/ml at 30°C with PC-TP (23 μg/ml) and a tenfold excess of unlabeled SUV (PC^{SUV}/PC^{OMV} molar ratio of 10). At the indicated time points OMV and SUV were separated by centrifugation for analysis as detailed in Section 2.

Table 2
Relative contribution of the CDP-choline pathway

	Specific radioactivity of choline[a] or PC[b] (10^3 dpm/nmol)	% PC originating from choline in growth medium
Growth medium	39.0[a]	–
Homogenate	7.4[b]	19
Mitochondria	7.2[b]	19
Microsomes	6.1[b]	16

The specific radioactivity of PC in several subcellular fractions was measured and related to the specific radioactivity of choline in the growth medium.

incorporation of choline in the medium in the PC of whole cells, mitochondria and microsomes is similar.

To study the kinetics and extent of the PC-TP mediated exchange process, OMV containing endogenous [^{14}C]PC synthesized via the CDP-choline pathway were incubated with an excess of SUV, labeled with the non-exchangeable marker [^{3}H]CE (PC^{SUV}/PC^{OMV} molar ratio of 10). The percentage of [^{14}C]PC transferred from the OMV to the SUV after different times of exchange with PC-TP is shown in Fig. 2. After approximately 15 min the equilibration of radiolabeled PC over the participating membranes is completed. The halftime of the exchange process is in the order of 1 min. After 20 min of exchange $88 \pm 3\%$ ($n = 3$) of the ^{14}C-labeled PC is transferred to the SUV. From this it was calculated as described in Section 2 that $99 \pm 4\%$ ($n = 3$) of the PC in the OMV is available for exchange by PC-TP under the conditions used. The exchangeable pool of PC was also determined for OMV isolated from cells grown on medium containing [^{3}H]methionine, in which case the labeling of PC occurs primarily via the methylation of PE. As above, the OMV were incubated with an excess of SUV, now labeled with the non-exchangeable marker [^{14}C]CE. Again it was found that all of the PC in the OMV is available for exchange by PC-TP. After 20 min of exchange $89.4 \pm 4\%$ ($n = 4$) of the ^{3}H-labeled PC is transferred to the SUV, which corresponds to an exchanged pool of $101 \pm 5\%$ ($n = 4$).

3.3. *Effect of pretreatment of OMV with proteinase K or sulfhydryl reagents on the extent of PC exchange*

The effect of proteinase K or sulfhydryl reagents on the size of the exchangeable pool was investigated. This was done by determining the extent of PC exchange in endogenous labeled OMV via the

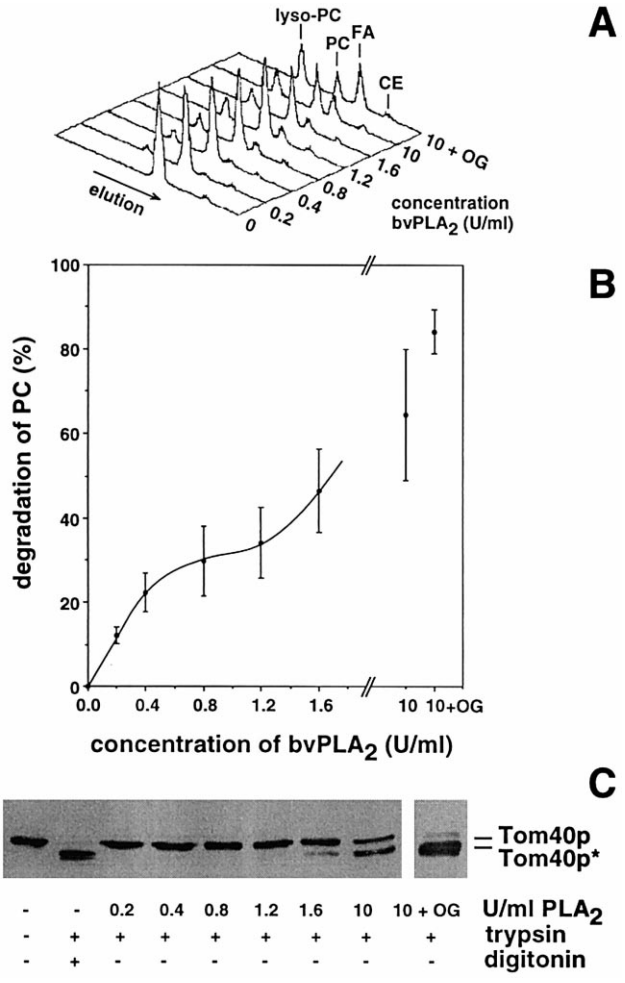

Fig. 3. The accessibility of newly introduced [^{14}C]DOPC in OMV to bvPLA$_2$ and the intactness of bvPLA$_2$-treated OMV. [^{14}C]DOPC was introduced into OMV by PC-TP from radiolabeled SUV for 20 min at 30°C. OMV were reisolated by centrifugation and incubated at a concentration of 0.6 mg/ml for 10 min at room temperature with bvPLA$_2$ at the indicated concentrations in the presence of 0.1 mM Ca^{2+}. Octylglucoside (OG, 40 mM) was added in control experiments to achieve maximal degradation of phospholipids by bvPLA$_2$. Phospholipase activity was inhibited by the addition of EGTA (1 mM). The OMV lipids were extracted and analyzed by HP-TLC. The integrity of the membranes after phospholipase treatment was checked by assessing the accessibility to trypsin of Tom40p. (A) Radioactivity scan of a TLC plate showing the distribution of the ^{14}C-label over PC, lysoPC, fatty acid (FA) after incubation with different concentrations of phospholipase. The [^{3}H]CE peak originates from the SUV contamination in the OMV pellet. (B) Quantification of the concentration dependent lipolysis of newly introduced [^{14}C]DOPC by bvPLA$_2$. The data have not been corrected for the ^{14}C-label present in the contaminating SUV, which was estimated to be approximately 10% of the total ^{14}C-label. The error bars represent the standard deviation ($n = 4$). (C) Assessment of the integrity of OMV after phospholipase treatment by analysis of the accessibility of Tom40p to trypsin. A typical Western blot is shown. The first two lanes show the untreated OMV and a control where Tom40p is completely accessible to trypsin after solubilization of the OMV with digitonin (0.2% (w/v)).

CDP-choline pathway as above, after a pretreatment with proteinase K or the sulfhydryl reagents NEM and PCMBS. The size of the PC pool in OMV available for exchange by PC-TP after 20 min was not significantly affected by any of these pretreatments (94.4 ± 1.4% (proteinase K), 96.3 ± 0.6% (NEM) and 96.8 ± 0.9% (PCMBS), $n = 3$ for all).

3.4. Introduction of synthetic radiolabeled PC into OMV by PC-TP and assessment of its accessibility to PLA_2

To determine whether in vitro introduced PC equilibrates over both leaflets of the OMV membrane, its transmembrane distribution was investigated. For this purpose, ^{14}C-labeled DOPC was introduced into unlabeled OMV by the action of PC-TP. To minimize the contamination of OMV with SUV, incubations were performed with approximately equal amounts of PC present in the populations of donor and acceptor vesicles (donor/acceptor ratio approximately 1). The depletion of ^{14}C-label from the SUV fraction is used to calculate the size of the exchanged pool in this experimental set-up. This results in lower accuracy of measurement, since the maximally possible relative depletion of label from the donor membranes strongly depends on the donor/acceptor ratio. It was calculated that at these low donor/acceptor ratios up to 80% ($n = 2$) of the PC present in the OMV had participated in the exchange after 20 min. The kinetics of the exchange were similar to those depicted in Fig. 2. The difference in calculated pool size (80% versus 100%) for synthetic and endogenous PC most likely is due to the experimental differences and concomitant technical limitations in the quantification of the exchanged pool size, and not to different behavior of the probes.

To determine whether the newly introduced [^{14}C]DOPC molecules equilibrate over both leaflets, the OMV which were reisolated after an incubation with PC-TP and [^{14}C]DOPC-labeled SUV were treated with phospholipase A_2 from bee venom ($bvPLA_2$) for 10 min. Fig. 3A shows the HP-TLC analysis of OMV phospholipid extracts from a typical experiment. In Fig. 3B the degradation of newly introduced [^{14}C]DOPC by increasing concentrations of $bvPLA_2$ is quantitated. Even though the variation between experiments was considerable as can be seen

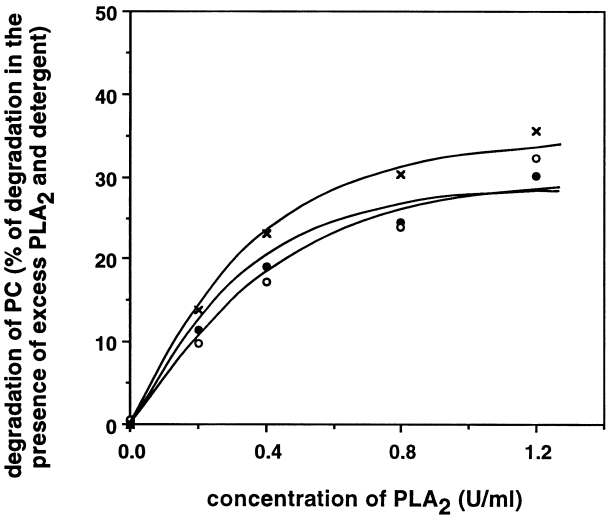

Fig. 4. Comparison of the degradation of newly introduced and endogenous PC by $bvPLA_2$ The degradation of radioactive PC, introduced by PC-TP (×) or synthesized in vivo via the CDP-choline pathway (●) or via methylation of PE (○), upon treating OMV with $bvPLA_2$ at the indicated concentrations is shown. The data is presented as percentage of the maximal degradation of the radiolabeled PC in the presence of excess PLA_2 (10 U/ml) and the detergent octylglucoside. [^{14}C]DOPC was introduced into OMV by PC-TP from radiolabeled SUV for 20 min at 30°C and the OMV were reisolated by centrifugation. OMV containing radiolabeled endogenously synthesized PC via either of the two biosynthesis pathways were prepared as described in Section 2. OMV were incubated at a concentration of 0.6 mg/ml for 10 min at room temperature with $bvPLA_2$ at the indicated concentrations in the presence of 0.1 mM Ca^{2+}. Phospholipase activity was inhibited by the addition of EGTA (1 mM). The OMV lipids were extracted and analyzed by HP-TLC. The degradation of PC in OMV containing endogenously synthesized radiolabeled PC by $bvPLA_2$ was calculated after correction for the minor amounts of lysoPC present in these vesicles in untreated controls.

from the error bars representing the standard deviation from four independent experiments, the same type of curve shape was obtained in each case. With increasing concentrations of phospholipase increasing amounts of [^{14}C]DOPC are degraded and a plateau value of 30–35% degradation is reached at 0.8 U PLA_2/ml which is followed by a sudden further increase at 1.6 U/ml. In the presence of an excess of phospholipase and the detergent octylglucoside, 85 ± 5% of the radiolabeled PC is degraded.

The intactness of the OMV after $bvPLA_2$ treatment was assessed by probing the accessibility of Tom40p to trypsin. Tom40p is not degraded by tryp-

sin when the mitochondrial outer membrane is intact [27]. The first two lanes in Fig. 3C show the intact Tom40p in untreated OMV and the appearance of the degradation product with a higher electrophoretic mobility upon treating the membrane with trypsin after solubilization with digitonin. Tom40p stays intact when OMV are treated with trypsin after they have been incubated with the phospholipase up to a concentration of 1.2 U/ml, confirming the sealed nature and right-side-out orientation of the OMV [15]. Only after treatment with a bvPLA$_2$ concentration of 1.6 U/ml and higher, the cleavage site for trypsin becomes exposed and a degradation product of Tom40p appears upon treatment with trypsin. In the presence of an excess of phospholipase and the detergent octylglucoside, Tom40p is virtually completely accessible to trypsin (Fig. 3C). To check whether the inaccessibility of Tom40p after phospholipase treatment reflects the closed nature of the membrane during the activity of the phospholipase, i.e., to rule out the possibility that during phospholipase treatment the membrane structure reorganizes itself allowing temporary access of the phospholipase to the lumen of the OMV after which closed structures are formed again, control experiments were performed in which FITC–dextran (M_r 20 000) was enclosed in the OMV lumen. These confirmed that leakage of the fluorophore from the lumen of the OMV only occurred under conditions where Tom40p could be degraded by trypsin (not shown). The sudden increase in the degradation of labeled PC accompanied by the loss of barrier function of the OMV membrane as shown by the accessibility of Tom40p to trypsin at 1.6 U/ml strongly suggest that at this concentration bvPLA$_2$ gains access to a PC pool located in the inner leaflet of the membrane, which remains protected against degradation while the membrane is still intact. These results imply that a portion of the newly introduced [^{14}C]DOPC has moved to the inner leaflet.

In order to compare the transmembrane distribution of newly introduced PC to the transmembrane distribution of endogenous PC, OMV containing [^{14}C]PC or [^{3}H]PC synthesized via the CDP-choline pathway or via the methylation of PE, respectively, were also treated with increasing concentrations of bvPLA$_2$. Fig. 4 shows that the accessibility to phospholipase of newly introduced and endogenous PC synthesized in vivo via either of the two biosynthetic pathways is very similar. From this it can be concluded that newly introduced PC adopts a transbilayer orientation similar to that of the endogenous PC, and that PC is not exclusively present in the outer leaflet of the OMV. These results and the fact that the PC in OMV is completely available for exchange by PC-TP demonstrate the occurrence of rapid bidirectional transbilayer movement of PC.

4. Discussion

The present findings demonstrate the occurrence of bidirectional transmembrane movement of both endogenous and in vitro introduced PC in isolated mitochondrial outer membrane vesicles from *S. cerevisiae*. This process is fast, with a $t_{1/2}$ of 1 min or less at 30°C, and causes newly introduced radiolabeled PC to adopt a transmembrane distribution similar to that of endogenous PC irrespective of its route of biosynthesis, i.e., via methylation of PE or via the CDP-choline pathway. The extent of the transmembrane movement is not influenced by pretreatment with protein-modifying agents.

In addition, the relative importance of the CDP-choline pathway to net PC biosynthesis was assessed by in vivo labeling. It was found that under the culture conditions used approximately 20% of total cellular PC originates from free choline present in the growth medium. However, as pointed out previously by other authors [6], this is probably an underestimate of the total contribution of the CDP-choline pathway to the production of PC since the choline originating from turnover of PC produced via methylation of PE can also be used for production of PC via the CDP-choline pathway. Comparison of the amount of choline incorporated into PC and the amount of choline originally present in the growth medium (not shown), suggests that the amount of choline in the medium is limiting its use for PC synthesis under the conditions used. Importantly, no significant differences in the contribution of choline from the medium for production of PC for the whole cell and the mitochondria were found. This result indicates no preference of the mitochondria for importing PC synthesized by either one of the pathways of biosynthesis.

The conclusions regarding the transmembrane movement of PC in the mitochondrial outer membrane are based on several observations. Upon extraction of radiolabeled PC from isolated OMV by PC-TP, 100% of the endogenous PC is available for exchange, with a halftime in the order of 1 min. In the reverse experiment where [^{14}C]DOPC was introduced into OMV, similar characteristics of the exchange process were found, demonstrating that the behavior of the synthetic phospholipid resembles that of the endogenous phosphatidylcholine.

The complete availability of PC in OMV for exchange by PC-TP could in principle be explained by an exclusive localization of PC in the outer leaflet of the membrane. However, such a complete asymmetry in the mitochondrial outer membrane was considered highly unlikely since it was never found for any other biological phospholipid bilayer and moreover, data from literature argue against it [28]. In the present study it was demonstrated that 30–35% of the [^{14}C]DOPC introduced in the outer membrane can be degraded by bvPLA$_2$ while the OMV are still intact. The sudden further increase in degradation of labeled PC accompanied by the loss of barrier function strongly suggests that the phospholipase then gains access to a PC pool located in the inner leaflet of the membrane which remains protected against degradation while the membrane is still intact. This is consistent with movement of part of the newly introduced PC to the inner leaflet. The maximal accessibility of PC to bvPLA$_2$ found in intact OMV should only be regarded as a relative measure of the transmembrane distribution and not as an absolute value. Most likely, the intactness of the OMV is affected before all of the phospholipids in the outer leaflet are completely degraded by the phospholipase, due to destabilization of the bilayer structure by the presence of large amounts of lysophospholipids and fatty acids. Nevertheless, the similar accessibility of newly introduced and endogenous PC to bvPLA$_2$ demonstrates that PC introduced by PC-TP assumes a transbilayer orientation similar to that of endogenous PC independent of its route of synthesis. This result in combination with the complete availability of the endogenous PC pool for exchange by PC-TP shows the bidirectional nature of the transmembrane movement and the similar behavior of the synthetic phospholipid and the endogenous PC. The transmembrane movement appears to be at least as fast as the PC-TP mediated exchange process with a halftime in the order of 1 min, since the data do not allow distinction between the rates of the actual exchange and the PC transmembrane movement.

The fact that the entire pool of PC in OMV is available for exchange by PC-TP whereas only a limited part of the PC is accessible to degradation by PLA$_2$ in intact OMV may seem paradoxical. The possibility that the presence of Ca^{2+} during phospholipase treatment affects the transmembrane movement was ruled out, since the extent of the exchange as judged from the size of the exchangeable pool after 10 or 20 min was not decreased in the presence of 0.1 mM Ca^{2+} (data not shown). Several other explanations are possible. One is that PC-TP has direct access to the inner leaflet of the OMV but this possibility is considered highly unlikely in view of the molecular size of PC-TP as argued previously [29]. Alternatively, PC-TP itself or in combination with OMV membrane components could somehow directly induce the rapid bidirectional transmembrane movement, allowing redistribution of newly introduced and endogenous PC over both leaflets of the OMV. This effect would be rather specific for OMV since there are no indications that PC-TP has this ability in other biomembranes [30,31] nor does it induce transmembrane movement in protein-free phospholipid membranes [16,17]. Another explanation would be that the actual insertion of the phospholipid in the mitochondrial outer membrane rather than the interaction of PC-TP with the membrane somehow triggers flipping of the phospholipid. Alternatively, the redistribution of PC over both leaflets of the OMV could be occurring continuously, regardless of whether PC molecules are inserted or extracted, by a non-stop process which is no longer occurring once the membrane structure is disturbed by hydrolysis of phospholipids or which does not transport the lysoPC molecules produced by the phospholipase.

The results obtained here on the transbilayer orientation of PC are in agreement with the conclusion drawn in a study by Sperka-Gottlieb et al. on the lipid topology in OMV from yeast [28], but at the same time conflict with the results regarding the size of the rapidly exchangeable pool of this phospholipid. They found that only approximately half of

the PC in OMV is available for rapid exchange, using a non-PC-specific lipid transfer protein, i.e., the phosphatidylinositol/phosphatidylcholine transfer protein (Sec14p). Extraction of the remaining PC proceeded at a slower rate which was interpreted as slow transbilayer movement of PC located in the inner leaflet ($t_{1/2} \sim 50$ min). However, the transport protein used by Sperka-Gottlieb et al. has a significantly higher relative affinity for phosphatidylinositol than for PC (for a review see [32]). This property and the fact that a different donor/acceptor system was used could explain the differences between the two studies.

Mitochondrial contact sites have often been suggested to be zones of intramitochondrial lipid translocation. A study by Lampl et al. [7] showed that, after introduction of radiolabeled PC by a non-PC-specific transfer protein into yeast mitochondria, the radiolabeled PC can be detected almost immediately in the outer membrane as well as in the contact site fraction and the inner membrane at similar specific radioactivities. The present study provides evidence that transport of PC across the outer membrane is not restricted to the contact sites. No conclusions can be drawn as to which step in the overall process of intramitochondrial PC translocation in yeast is rate limiting. Previous work from our laboratory with rat liver OMV and mitochondria strongly suggests that the transmembrane movement across the mitochondrial outer membrane is not rate limiting [18,28]. Considering the short doubling time of the yeast *S. cerevisiae*, in vivo translocation of phospholipids to and in the mitochondrion has to be very fast to ensure simultaneous development with the rest of the cell. A high rate of PC translocation between endoplasmic reticulum and mitochondria in vivo was demonstrated in pulse-chase studies [8]. The short halftime found for transmembrane movement of PC across the mitochondrial outer membrane from yeast in vitro is in good agreement with the in vivo requirements.

The rate of transmembrane movement of PC in OMV found in the present study would suggest the involvement of proteins in this process, since in protein-free model membranes only very slow transbilayer movement occurs (e.g., [17]). As demonstrated by other authors, several phospholipid transport processes (both intermembrane and transmembrane) in eukaryotic cells require a source of energy and/or are sensitive to protein-modifying agents [33–36], which is indicative of a protein-mediated process, and in some cases proteins capable of catalyzing such transport processes were also identified [37–39]. However, the extent of the transmembrane movement of PC in the OMV was not influenced by pretreatment with proteinase K, NEM, or PCMBS, which provides no direct clues for the involvement of proteinaceous factors. It is considered unlikely that the process of transmembrane movement demonstrated in the present study has a strict energy requirement since all the experiments described were performed without addition of any possible sources of energy. This is in agreement with observations by other authors that energy depletion did not significantly inhibit the import of phosphatidylcholine into intact mitochondria in vitro [7]. Other membranes that were reported to display energy-independent rapid phospholipid transmembrane movement are the endoplasmic reticulum from rat liver [16], the inner membrane of *Escherichia coli* [40], and the membrane of *Bacillus megaterium* [41]. The present study shows for the first time the occurrence of rapid transmembrane movement of a phospholipid in an intracellular membrane (i.e., the mitochondrial outer membrane) from the yeast *S. cerevisiae*. Rapid PC flip-flop of in vitro introduced synthetic PC was previously shown to occur in isolated mitochondrial outer membranes from rat liver [29]. This validates the use of yeast as a model eukaryote for studying the mechanisms of lipid transport across intracellular membranes. Its sequenced genome and potential for classical genetic studies and genetic engineering unequaled in the eukaryotic world render yeast the eukaryote of choice for the eventual elucidation of the putative proteinaceous factors governing these processes. This study provides the basis for future research in that direction.

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