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Intramitochondrial distribution and transport of phosphatidylserine and its decarboxylation product, phosphatidylethanolamine. Application of pyrene-labeled species

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To investigate the mechanism of intramitochondrial translocation of phosphatidylserine and its decarboxylation product, phosphatidylethanolamine, the distribution of these lipids between the outer (OM) and inner (IM) mitochondrial membranes, as well as their transversal and lateral distribution in OM were studied. Fluorescent, pyrenyl derivatives of phosphatidylserine (Pyr_xPS) and phosphatidylethanolamine (Pyr_xPE) species were employed because they allow: (i), direct monitoring of PS (and PE) loading to the mitochondria; (ii), assay of PS decarboxylation by high-performance liquid chromatography with fluorescence detection and (iii), determination of the lateral distributions of PS and PE within the mitochondrial membranes. All Pyr_xPS species tested were efficiently decarboxylated by the solubilized decarboxylase and thus the distribution of the endogenous PE could be also studied. When the Pyr_xPS species were loaded to isolated mitochondria very little, if any, of the loaded Pyr_xPS or of the Pyr_xPE product was found in IM independent of the time and temperature of incubation, strongly suggesting that these lipids either never enter IM or their residence there is only transient. When mitochondria preloaded with Pyr_4PS were incubated with an excess of acceptor vesicles in the presence of the lipid transfer protein, 80% of Pyr_4PS and 30–40% of the Pyr_4PE product were transported to the acceptor vesicles, indicating that at least corresponding fractions of these lipid were located in, or were in rapid equilibrium with the outer leaflet of OM. Since the decarboxylase is located in the inner membrane, these results signify that both PS and PE must be able to move readily across OM. Determination of the excimer to monomer ratio as the function of pyrenyl lipid concentration in mitochondria (i.e., OM) gave parallel results for Pyr_xPS and -PE species suggesting the lateral distribution of PS and PE in OM is similar and thus there is no specific enrichment of PS to the contact sites. To investigate the mechanism of PS transport from the outer leaflet to the decarboxylation site, the influence of Pyr_xPS hydrophobicity, i.e., pyrenylacyl chain length, on the rate of decarboxylation was determined. The variation of the length of the pyrenyl acyl chain from 4 to 12 carbons did not significantly affect the rate of Pyr_xPS decarboxylation in intact mitochondria, indicating that the transport of PS from the outer leaflet of OM to the site of decarboxylation takes place by lateral diffusion rather than by spontaneous or protein-mediated transport. The implications of these findings on the mechanism of intramitochondrial transport of PS and PE are discussed in terms of alternative models.

Introduction

In some mammalian cells most, if not all, of phosphatidylethanolamine (PE), a major membrane component, is produced by decarboxylation of endoplasmic reticulum (ER) derived phosphatidylserine (PS) by an enzyme located in the inner mitochondrial membrane [1,2]. After the decarboxylation event the PE product is exported to ER and other intracellular organelles [3,4]. Thus, apparently two intercompartment transport phases are involved in the translocation of both PS and PE: one between the ER and the outer mitochondrial membrane (OM) and the other between OM and the inner mitochondrial membrane (IM). It has been shown recently by Voelker [5] that the transport of PS from the ER to the mitochondria may in fact consist of two (undefined) steps, the first of which is ATP-dependent and the second which is ATP (i.e., energy) independent.

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Abbreviations: IM, inner mitochondrial membrane; OM, outer mitochondrial membrane; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Pyr_xPS, 1-palmitoyl-2-(1-pyrenylacyl)$_x$-*sn*-glycero-3-phosphoserine, x indicates the number of carbons in the aliphatic chain; Pyr_xPE, 1-palmitoyl-2-(1-pyrenylacyl)$_x$-*sn*-glycero-3-phosphoethanolamine; POPC, 1-palmitoyl-2-oleyl-phosphatidylcholine; DOPE, 1,2-dioleoyl-phoshatidylethanolamine; BBPS, bovine brain phosphatidylserine; TNP-PE, 2,4,6-trinitrophenylphosphatidylethanolamine; nsL-TP, non-specific lipid transfer protein.

Concerning the intramitochondrial transport of PS and PE it has been recently suggested that the contact sites between OM and IM are intimately involved in the transport of PS and PE [6–8]. This suggestion was based on the findings that radioactive PS imported from microsomal vesicles in vitro was found to be enriched to the isolated contact site fractions of rat liver mitochondria [6]. The important role of contact sites is also supported by the inhibition of PS decarboxylation by dinitrophenol [9], since this compound is known to decrease the number of these sites [10,11]. On the other hand, no specific enrichment of PS decarboxylase to the contact sites of yeast or rat liver mitochondria has been found [8,9].

The mechanism of intramitochondrial PS and PE transport, through the contact sites or otherwise, between OM and IM is not clear. To study this, we have employed fluorescent, pyrenyl PS (Pyr_xPS) and PE (Pyr_xPE), species. These derivatives could be introduced efficiently to mitochondria by protein-mediated transfer from lipid vesicles and were used to study the distribution of PS and PE between OM and IM, over OM and in the plain of OM. In addition, information on the mechanism(s) of PS transport from OM to the site of decarboxylation was obtained by determining the effect of the Pyr_xPS hydrophobicity on the rate of decarboxylation. The results are discussed in terms of alternative models of intramitochondrial lipid transport.

Materials and Methods

Lipids

Phosphatidylserine species containing a palmitoyl chain in the *sn*-1 position and a pyrenylacyl chain of variable length in the *sn*-2 position were prepared from the respective PC species [12] by using phospholipase D (*Streptomyces species*) mediated transphosphatidylation in the presence of L-serine as described by Comfurius et al. [13]. The Pyr_xPS species were purified by HPLC on a silica column (8 × 250 mm) eluted with a gradient of CH_3OH/0.1% H_2SO_4 (9:1 (v/v)) to $CHCl_3$. The sulfuric acid was removed by partitioning [14] and repeated washing of the lower phase with CH_3OH/H_2O (1:1). The lower, $CHCl_3$ phase was then made slightly basic by adding dilute NH_4OH in methanol, evaporated to dryness under nitrogen and the lipid was dissolved to $CHCl_3/CH_3OH$ (9:1 (v/v)). Pyr_xPE species were prepared analogously. 1-Palmitoyl-2-oleyl-phosphatidylcholine (POPC), 1,2-dioleylphosphatidylethanolamine (DOPE) and *N*-trinitrophenylphosphatidylethanolamine (TNP-PE) were supplied by Avanti Polar Lipids (Birmingham, AL, USA), whereas bovine brain phosphatidylserine (BBPS) was obtained from Sigma and glycerol tri[1-^{14}C]oleate ([^{14}C]triolein, spec. act. 1.85–2.2 GBq/mmol) from Amersham International (Amersham, UK). All lipids were homogeneous on TLC and were stored at < −20°C.

Biological materials

Solubilized rat liver phosphatidylserine decarboxylase was obtained as previously described [15] and was kindly provided by Ms. A. Dygas from the Nencki Institute of Experimental Biology. Mitochondria were obtained from livers of adult Wistar rats by the procedures described earlier [16,17]. The latency of cytochrome-*c* oxidase in the isolated mitochondria was 93.6 ± 0.3, as determined spectrophotometrically [18]. Thus, a great majority of the mitochondria maintained their integrity under these conditions. The non-specific lipid transfer protein (nsL-TP) from bovine liver (spec. act. 5180 nmol phospholipid/h per mg protein) was kindly provided by Prof. K.W.A. Wirtz (State University of Utrecht, The Netherlands). The protein was stored at < −20°C in a buffer containing 50% glycerol.

Assay of Pyr_xPS decarboxylation by solubilized enzyme

Pyr_xPS (0.5 nmol) was blown to dryness and kept under vacuum for 1 h. Buffer containing 100 mM sodium phosphate (pH 7.4), 10 mM DTT, 1 mM EDTA, 1.5 mM Triton X-100 was then added and the dispersion was equilibrated for 5 min at 37°C. Solubilized enzyme (0.2 mg) was included and the incubation was carried out in the dark at 37°C with continuous shaking in a total volume of 0.1 ml. The reaction was terminated by addition of 0.67 ml CH_3OH, 1.33 ml $CHCl_3$ and 0.4 ml 0.2 M HCl. After separation of the phases, the lower phase was washed with the mixture of $CHCl_3/CH_3OH$/0.2 M HCl (3:48:47 (v/v)) and $CHCl_3:CH_3OH:H_2O$ (3:48:47 (v/v)), evaporated and dissolved in 40 μl chloroform for HPLC analysis. Pilot experiments with Pyr_6PS showed that the amount of Pyr_6PE formed increased linearly up to at least 0.2 mg of mitochondrial protein per 0.1-ml incubation volume under the standard assay conditions (30 min incubation at 37°C; not shown). This protein concentration was therefore used for all Pyr_xPS species.

Determination of Pyr_xPS and Pyr_xPE by HPLC

An LKB 2249 instrument equipped with Spherisorb 4.6 × 100 mm silica column (PhaseSep, Deeside, UK), a Merck-Hitachi F1050 fluorescence detector and a Merck-Hitachi D2000 integrator was used to separate and quantitate Pyr_xPS and -PE. The solvent consisted of hexane/2-propanol/chloroform/5 mM L-serine-ethanolamine (pH 6.5)/tetrahydrofuran and a 5 min gradient (adapted from Ref. 19) from 38:47:10:5:0.5 to 32:50:10:8:0.3 (v/v) was run to separate PE and PS (retention times 1.8 and 3.0 min, respectively). All solvents were of HPLC grade. The recovery of total

pyrene lipid fluorescence was independent of the Pyr_xPS/Pyr_xPE ratio of the sample indicating that no specific losses of either lipid occurred upon extraction or HPLC.

Loading of mitochondria with Pyr_xPS and -PE species

Donor vesicles consisting of either Pyr_xPS/DOPE/TNP-PE/POPC or Pyr_xPE/BBPS/TNP-PE/POPC (5:5:10:80, molar ratio) and [^{14}C]triolein were prepared by dispersing the vacuum-dried lipid mixtures in a buffer (A) containing 220 mM mannitol, 70 mM sucrose, 2 mM Hepes, 0.5 mM EDTA and 0.5 mM EGTA (pH 7.4), followed by ultrasonication for 5 min (10 s on, 10 s off) on ice using Branson probe sonifier at 60 W output. The vesicle suspension was centrifuged at $15000 \times g$ for 10 min to remove any undispersed lipid and probe particles. Mitochondria (1 mg protein; approx. 200 nmol phospholipid), donor vesicles (10 nmol of total phospholipid) and nsL-TP (5–10 μg) were then incubated in 0.3 ml of buffer A for 10–20 min on ice to accomplish loading of the pyrenyl lipid to mitochondria. The increase of pyrene monomer fluorescence intensity resulting from the transfer of pyrenyl lipid molecules from the quenched donor vesicles to mitochondria was recorded to monitor the progress of the transfer process [12,20]. When the intensity leveled off, indicating that equilibrium had been reached, the mitochondria were isolated by pelleting in a table-top centrifuge (15000 rpm, 5 min) at 4°C. To assess the amount of probe transferred mitochondria an aliquot was solubilized with Triton X-100 (0.5%, final concentration) and the pyrene monomer fluorescence intensity was then measured and compared with the value obtained for the donor vesicles treated analogously. The amount of donor vesicles co-sedimenting with the mitochondria was determined from the distribution of the radioactivity of [^{14}C]triolein, a nonexchangeable donor vesicle marker, between the mitochondrial pellet and the supernatant.

Distribution of Pyr_xPS and Pyr_xPE between the outer and inner membranes and over the outer membrane

To determine the distribution of preloaded $Pyr_{10}PS$ and $Pyr_{10}PE$ between membranes, the mitochondria were treated with digitonin (0.3 mg/mg protein) and separated to OM and IM fractions exactly as described by Hovius et al. [16]. In agreement with their data, practically all of cytochrome-*c* oxidase, an inner membrane marker enzyme, was found in the pellet after solubilization of the outer membrane by digitonin. Triton X-100 (final concentration 0.5%) was then added and the pyrene monomer fluorescence intensity was determined and used, after subtraction of blanks obtained with unlabeled membranes, to calculate the distribution of the labeled lipids between OM and IM.

The distribution of Pyr_4PS and the Pyr_4PE product over the outer membrane was determined by subjecting preloaded mitochondria to a back-exchange procedure. The mitochondria (approx. 200 nmol phospholipid) were incubated with nsL-TP (10 μg) and an excess of acceptor vesicles (2000 nmol phospholipid) consisting of POPC, BBPS (95:5, molar ratio) and a trace amount of [^{14}C]triolein, the nonexchangeable donor marker, for 10 min at 30°C followed by sedimentation of the mitochondria as described above. Aliquots of the mitochondrial pellet and the supernatant containing the acceptor vesicles were then assayed for the Pyr_4PS and Pyr_4PE contents by HPLC as well as for the ^{14}C radioactivity. The amount of each pyrenyl lipid transported to the acceptor vesicles was then calculated after correction for co-sedimentation of a small fraction (about 5%) of the acceptor vesicles with the mitochondria. Pyr_4PS was used in this studies, since it and the Pyr_4PE produced thereof are the best substrates for nsL-TP (see below) and are thus more readily equilibrated between the mitochondria and the acceptor vesicles than their long-chain counterparts.

Fluorescence measurements

All fluorescence measurements were carried out on a Hitachi F-4000 spectrofluorometer equipped with a thermostated cuvette holder using quartz microcuvettes with 4 mm internal light path. The excitation was set to 344 nm and emission to 378 nm, respectively.

Other procedures

The concentration of the pyrenyl lipids was measured spectrophotometrically in chloroform using $E_{345} = 40000\ M^{-1}cm^{-1}$. The concentrations of the unlabeled lipids were determined by a phosphate assay [21]. Protein was determined using commercially available Bio-Rad Protein Assay [22]. Radioactivity was measured by liquid scintillation counting.

Results

Decarboxylation of Pyr_xPS species by solubilized enzyme

A prerequisite for the efficient use of Pyr_xPS species to investigate the intramitochondrial transport of PS and endogenous PE is that the Pyr_xPS species serve as substrates for the PS decarboxylase. This was studied by incubating the various Pyr_xPS species with the solubilized enzyme followed by extraction and analysis of the pyrenyl lipids by HPLC as described above. As depicted in Fig. 1A, all Pyr_xPS species were efficiently converted to corresponding PE species, thus demonstrating that the pyrenyl moiety does not prevent the interaction of the lipid with the active site of the enzyme. Interestingly, however, the initial rate of decarboxylation increased with the length of the pyrenylacyl chain so that the rate obtained for $Pyr_{14}PS$ was approx. 2-times higher than that for Pyr_4PS (Fig.

1B). The reasons for the slower rates observed for the short chain species is not clear, but could relate either to impaired binding to the active site of the decarboxylase, or alternatively to their (relative) exclusion from the enzyme-lipid boundary. Evidence for relative exclusion of the short chain pyrenyl lipids from a protein-lipid boundary has been obtained previously [23].

Loading of mitochondria with Pyr_xPS and -PE species

To test if Pyr_xPS (and -PE) species could be loaded to mitochondria by the non-specific lipid transfer protein (nsL-TP) we employed a previously developed assay which is based on dequenching of pyrene monomer fluorescence upon transfer of the pyrenyl lipid from quenched donor vesicles to unquenched acceptors [12,20]. When mitochondria were added to suspension of quenched donor vesicles containing 5 mol% of $Pyr_{10}PS$ or $Pyr_{10}PE$, sluggish increase of the pyrene fluorescence was observed, indicating slow spontaneous transfer of the probe to mitochondria was taking place (Fig. 2). However, when 10 μg of non-specific lipid transfer protein was added, very fast increase of fluorescence was observed, indicating much more efficient incorporation of the probe to mitochondria. Transport of the pyrenyl lipid to the mitochondria was confirmed by separating the mitochondria from the donor vesicles by centrifugation. Approx. 40–50% of $Pyr_{10}PS$ and 30–40% of $Pyr_{10}PE$ initially present in the donor vesicles was found in the mitochondrial pellet while only 3–6% of the [^{14}C]triolein, a non-exchangeable vesicle marker, was found in this pellet. Also other Pyr_xPS and Pyr_xPE species could be loaded to mitochondria (not shown), although the initial rate of incorporation decreased somewhat

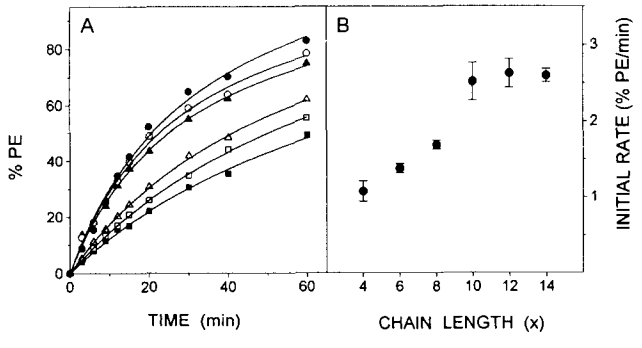

Fig. 1. Decarboxylation of Pyr_xPS species by solubilized phosphatidylserine decarboxylase. Each Pyr_xPS species (0.5 nmol) was incubated with solubilized enzyme (0.2 mg protein) and the amount of Pyr_xPE was determined as detailed in Materials and Methods. Panel A: Time dependence of Pyr_xPS decarboxylation. $x = 4$ (■), 6 (□), 8 (△), 10 (▲), 12 (○) and 14 (●). Panel B: The initial rate of decarboxylation as a function of the pyrenylacyl chain length (×). Bars represent the error of the determination of the initial rates (slopes) by regression analysis. The data are a compilation from three independent experiments each including all species.

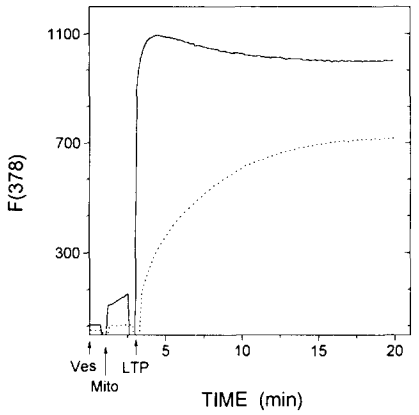

Fig. 2. Loading of Pyr_xPS and -PE to mitochondria. $Pyr_{10}PS$/DOPE/TNP-PE/POPC (solid line) or $Pyr_{10}PE$/BBPS/TNP-PE/POPC (dashed line) donor vesicles (Ves) were added to the fluorimeter cuvette (at 6°C) followed by mitochondria (Mito) and 10 μg of nsL-TP (LTP) and the pyrene monomer fluorescence intensity at 378 nm was monitored.

with increasing length of the pyrenylacyl chain as expected for the transfer protein used [24]. Only 2–6% of the Pyr_xPS species was converted to Pyr_xPE during the loading process if carried out at 0°C. Thus, the procedure allows facile introduction of Pyr_xPS species to mitochondria with only minor decarboxylation. The amount of Pyr_xPS species introduced to mitochondria represented less than 0.1% of total mitochondrial lipids which makes any significant perturbation of mitochondrial membranes very unlikely.

The faster rate of $Pyr_{10}PS$ transfer, as compared to $Pyr_{10}PE$, is most likely due to facts that (1) PS is more hydrophilic than PE, as indicated by its faster spontaneous transfer [25] and (2) the rate of nsL-TP-mediated lipid transport correlates positively with lipid hydrophilicity [24,26].

Distribution of $Pyr_{10}PS$ and the $Pyr_{10}PE$ product between the outer and inner mitochondrial membranes

$Pyr_{10}PS$ was loaded to mitochondria at 0°C as described above and the mitochondria were then incubated at 30°C for 0–60 min, resulting in 50% decarboxylation of $Pyr_{10}PS$ at 60 min. The OM and IM fractions were then isolated and analyzed for the $Pyr_{10}PS$ and $Pyr_{10}PE$ contents as described in Materials and Methods. As shown in Fig. 3, only 2–3% of the loaded $Pyr_{10}PS$ and 4–6% the $Pyr_{10}PE$ produced thereof were present in the IM fraction. Taking into account that a few percent of OM cofractionates with IM [16], it is obvious that both the loaded $Pyr_{10}PS$ and its decarboxylation product are almost completely restricted to OM and this distribution is stable up to at least 60 min after loading. Similar results were obtained when the post-loading incubation was carried out at 10, 20 or 37°C (not shown) indicating that the distribution is not temperature sensitive.

When $Pyr_{10}PE$ was loaded to mitochondria and the OM and IM fractions were analyzed, practically all (> 95%) of this exogenous $Pyr_{10}PE$ was found to be present in the OM fraction, independent of the loading temperature (not shown). Thus, the OM/IM distribution of PE does not depend on whether it is produced within mitochondria or is transported from donor vesicles by nsL-TP.

We note that the hydrophobicities of the pyrenyl lipids used in this experiment are similar to the average natural species [12] and thus the possibility that they would redistribute between OM and IM during the isolation of the corresponding fractions seems remote.

Distribution of PS and PE over the outer membrane

To study the transversal topology of the imported PS and the PE product in OM, Pyr_4PS was loaded to mitochondria at 0°C according to the standard protocol, the mitochondria were incubated for 0–40 min at 30°C to allow for formation of Pyr_4PE and were then subjected to back-exchange in the presence of nsL-TP and an excess of acceptor vesicles. As shown in Fig. 4A (closed symbols), 80% of Pyr_4PS was transported to the acceptor vesicles during the back-exchange process indicating that most of the Pyr_4PS molecules were in the outer leaflet of OM or in a pool (such as the inner leaflet of OM) which is in rapid equilibrium with the outer leaflet. The back-exchangeable Pyr_4PS fraction did not vary during the 40-min incubation period despite that the PE/PS ratio increased almost 50-fold (Fig. 4B), indicating that PS distribution over OM is independent of this ratio.

Of the Pyr_4PE produced in the mitochondria 30–40% was transported to the acceptor vesicles during

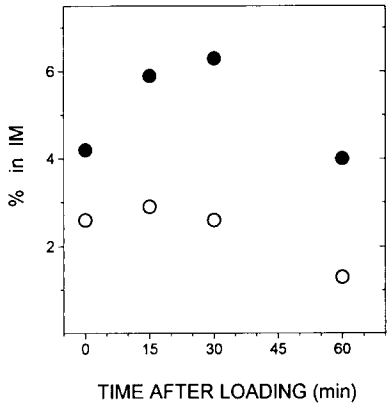

Fig. 3. Distribution of $Pyr_{10}PS$ and the PE product between the outer and inner mitochondrial membranes. Mitochondria preloaded with $Pyr_{10}PS$ were incubated for the indicated times at 30°C and then treated with digitonin to obtain OM and IM fractions (see Materials and Methods). The amount of $Pyr_{10}PS$ (○) and the $Pyr_{10}PE$ product (●) in OM and IM were then determined by HPLC analysis and the percentage in IM plotted as a function of time. Essentially identical results were obtained when the postloading incubation was carried out at 10, 20 or 37°C.

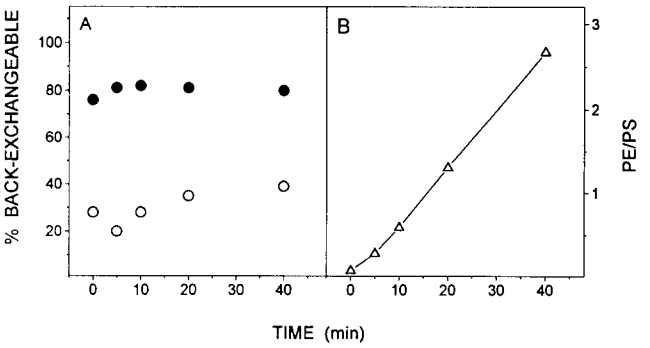

Fig. 4. Exposure of Pyr_4PS and -PE on the outer surface of OM. Mitochondria (1 mg protein) loaded with Pyr_4PS were incubated for various times at 30°C and then subjected to a back-exchange procedure, i.e., 10 min incubation in the presence of acceptors vesicles (2000 nmol) and 10 μg nsL-TP (see Materials and Methods). (A) The fraction of Pyr_4PS (●) and the Pyr_4PE product (○) transported to the acceptor vesicles was assayed as outlined in Materials and Methods. The ratio of PE to PS in combined mitochondria and acceptor fractions is plotted in panel B.

the back-exchange process (Fig. 4A). While these figures would suggest that the fraction of PE present in (or having access to) the outer leaflet of OM is considerably smaller than that of PS (40 vs. 80%), it appears that the exposed Pyr_4PE fraction is underestimated due to incomplete depletion during the 10 min back-exchange period. This was proven by control experiments (not shown) where the pyrene excimer to monomer intensity ratio (E/M), which is proportional to the local concentration of a pyrenyl lipid (see below), was monitored continuously during back-exchange of preloaded Pyr_4PS and -PE from hydroxylamine-treated (to inhibit Pyr_4PS decarboxylation, see below) mitochondria. In case of Pyr_4PS the decrease of E/M had almost leveled off to a value less than 10% of the original one already 10 min after addition of nsL-TP, indicating rapid equilibration of this lipid between the mitochondria and the acceptor vesicles. In contrast, E/M was still decreasing at 10 min in the case of Pyr_4PE indicating that back-exchange of Pyr_4PE was not yet complete. This slower equilibration of Pyr_4PE is in accordance with the inherently slower transfer of PE as compared to PS (Fig. 2). While more extensive depletion of Pyr_4PE from the exposed pool could have been achieved by extending the time of back-exchange, this was not done to avoid rupture of the outer membrane [27] which would severely compromise the results. Based on these control experiments we conclude that at the amount of Pyr_4PE exposed to the outer surface of OM is considerably higher (probably over 50–60%) than indicated by the data in Fig. 4A.

It is relevant to note here that nsL-TP very likely does not have access to the inner leaflet of OM, since both its molecular mass (12.4 kDa) and overall charge are similar to those of cytochrome *c* which has been shown to be unable to pass through OM [28,29].

Lateral distribution of Pyr_xPS and Pyr_xPE in the outer mitochondrial membrane

Ardail et al. [6,7] have recently found that radiolabeled PS imported to mitochondria from microsomal vesicles in vitro, but not the PE produced in mitochondria, was markedly enriched to contact site fractions isolated from mitochondria. Pyrenyl lipids offer the possibility to study whether such local enrichment of PS is indeed taking place. This is based on their ability to form s.c. excimers and on the fact that the fraction of molecules forming excimers (and thus emitting excimer fluorescence centered at 475 nm) is proportional to the average distance between the pyrenyl lipid molecules, i.e., their local surface density [25,30,31]. Accordingly, segregation of a pyrenyl lipid to certain domains, such as the contact sites, would result in a higher excimer to monomer intensity ratio as compared to a pyrenyl lipid not experiencing such a segregation. When Pyr_4- or Pyr_{10}-derivatives of PS or PE were loaded to mitochondria (pretreated with hydroxylamine to prevent Pyr_xPS decarboxylation) the E/M vs. pyrene lipid concentration plots were virtually identical for PS and PE independent whether the Pyr_4 or Pyr_{10} derivatives were used (Fig. 5), strongly suggesting that the lateral distribution modes of Pyr_xPS and Pyr_xPE in OM were similar. Thus, if enrichment of PS to the OM/IM contact sites indeed takes place, PE should be similarly enriched to these sites.

Effect of Pyr_xPS hydrophobicity on its rate of decarboxylation in intact mitochondria

Useful information of the mechanisms of intermembrane lipid translocation can be obtained by studying the effect of lipid acyl chain length (i.e., hydrophobicity) on the rate of intermembrane transport [32,33]. The rate of transport of PS from the site of insertion to the site of decarboxylation can be determined, if transport rather than decarboxylation is rate limiting. Accordingly, Pyr_xPS species with a labeled chain of variable length were loaded to mitochondria at 0°C and the initial rates of decarboxylation were determined after shifting the temperature to 30°C. As shown in Fig. 6, the rate of decarboxylation did not vary significantly when the length of the pyrenylacyl chain was varied from 4 to 12 carbons. This strongly suggests that neither spontaneous nor protein mediated transfer, both of which are strongly dependent on lipid hydrophobicity [12,24,32,34] through the aqueous intermembrane space is involved in the translocation process. Lateral diffusion, which is not markedly dependent on the acyl chain length [35], thus remains as the most likely mechanism for the intramitochondrial PS transport. The assumption that transport, not decarboxylation, is rate limiting is supported by the fact that the effect of pyrenylacyl chain length on the rate of Pyr_xPS decarboxylation in the micellar system (Fig. 1B), where transport is obviously not rate limiting, was quite different from that found for intact mitochondria (Fig. 6). We note also that Pyr_xPS introduced to mitochondria represented always $< 0.1\%$ of total mitochondrial phospholipids thus making the saturation of the decarboxylase unlikely.

Deviating from the general tendency the rate of decarboxylation of $Pyr_{14}PS$ was low. We have no obvious explanation for this. A possibility is that the excessive length (equal to that of a saturated C_{20}-acyl chain) of the Pyr_{14}-chain results in anomalous behavior such as slow transbilayer movement, or in preferential parti-

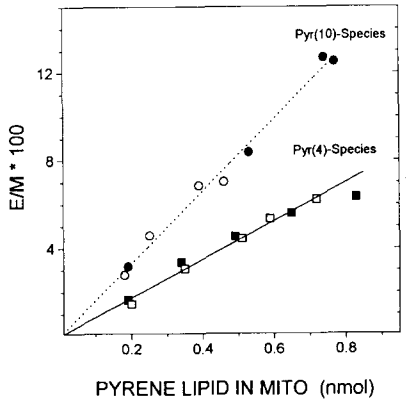

Fig. 5. Lateral distribution of Pyr_xPS and Pyr_xPE in the outer mitochondrial membrane. Pyr_4PS (■), Pyr_4PE (□), $Pyr_{10}PS$ (●) or $Pyr_{10}PE$ (○) was loaded to mitochondria pretreated with 25 mM hydroxylamine, an irreversible inhibitor of PS decarboxylase [59] for 30 min at 10°C. Pyrene excimer (E) and monomer (M) intensities were then determined and the E/M ratio plotted against the amount of pyrenyl lipid incorporated to mitochondria. HPLC analysis of the Pyr_xPS-labeled mitochondria after E/M measurements showed less than 5% decarboxylation.

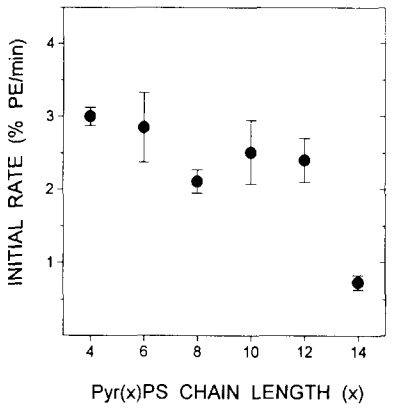

Fig. 6. Effect of the length of pyrenylacyl chain on the rate of intramitochondrial transport of Pyr_xPS species. Mitochondria (1 mg protein) were loaded with one of the Pyr_xPS species according the standard protocol, incubated at 30°C and analyzed for the content of Pyr_xPS and Pyr_xPE as a function of time. The rates of decarboxylation represent the initial slopes obtained by fitting to data from two independent experiments both including all species. The standard deviations of fitting are shown.

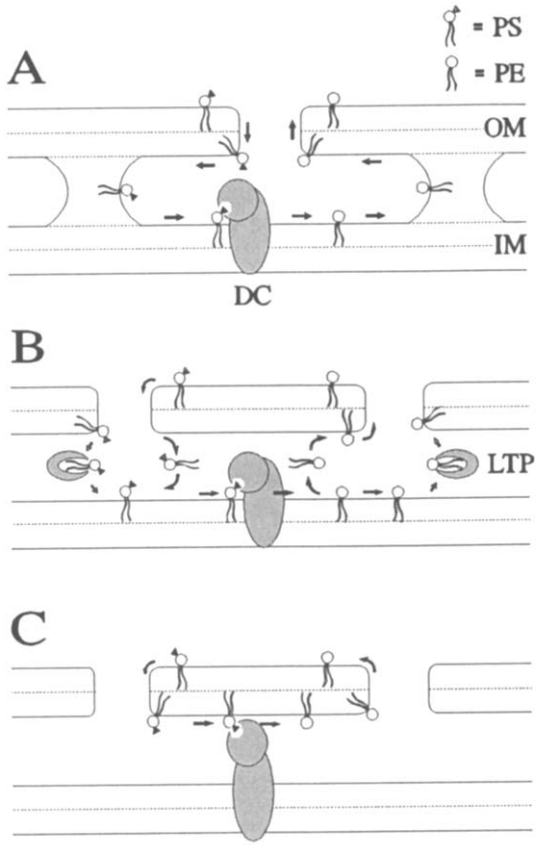

Fig. 7. Models for the intramitochondrial transport of PS and PE. According to model A, lipids move between OM and IM by lateral diffusion through s.c. semi-fusion sites bridging the two membranes. In model B this movement is accounted for by either lipid transfer proteins (LTP) or by spontaneous diffusion through the intermembrane space. Model C implies that the decarboxylation takes place while PS remains in the inner leaflet of the outer membrane. The pores drawn to OM may correspond those known to allow the pass-through of various kinds of small molecules. OM, outer membrane; IM, inner membrane, DC, phosphatidylserine decarboxylase; LTP, lipid transfer protein.

tioning of $Pyr_{14}PS$ to some (rigid) lipid domains or protein boundaries thus slowing down its lateral diffusion.

Discussion

Models for intramitochondrial PS and PE transport

A particularly intriguing question related to intramitochondrial lipid transport is: How can ER-derived PS be decarboxylated by an enzyme located in the inner mitochondrial membrane without mixing of the PE product with the inner membrane PE pool(s) [9,36]? To visualize this and other problems related to intramitochondrial PS and PE transport three alternative models are shown in Fig. 7. The compatibility of each model with the present as well as relevant previous findings is discussed in the following.

According to Model A, PS transported from ER to the outer leaflet of OM first moves to the inner leaflet through hypothetical pores ('flip sites') and then diffuses further to the inner membrane along 'lipidic bridges' joining the two membranes, and is finally decarboxylated by the enzyme located in this membrane. The PE produced can then diffuse back to the outer monolayer of OM along a reverse route. The 'lipidic bridges', which may be analogous to the s.c. membrane contact sites, represent the key elements of this model, since they (i) allow facile diffusion of PS and PE between OM and IM and (ii) prevent (assuming they have an annular shape) mixing of these lipids with those in IM proper. Such bridges could form by semi-fusion of OM and IM [35]. Evidence for the existence of such semi-fusion or contact sites in mitochondria has been obtained by electron microscopy [10,37]. In addition, the high concentrations of cardiolipin and PE in contact-site-enriched fractions [8,38] and the tendency of these lipids to form nonbilayer structures [39] provide circumstantial evidence for the occurrence of such sites. Voelker has reported that doxorubicin (adriamycin) markedly inhibits PS decarboxylation in permeabilized cells [40]. Since doxorubicin had been reported to have a high affinity specifically for cardiolipin containing membranes [41,42], it was proposed that this compound blocks the intramitochondrial transport of PS [40], possibly by perturbing the structure of contact sites. However, we have been unable to detect any reproducible inhibition of Pyr_xPS decarboxylation by doxorubicin added to mitochondria independent of the Pyr_xPS species used or whether spontaneous insertion, protein-mediated transport or Ca^{2+}-mediated fusion [43] was used for loading of the species to mitochondria (results not shown). As similar results were recently obtained with yeast mitochondria [44] it appears that doxorubicin does not inhibit intramitochondrial transport of PS. Thus, the inhibitory effect observed for permeabilized cells is probably related to the transport of PS from ER to mitochondria. In this respect, it is relevant to note that more recent binding studies have showed that the affinity of doxorubicin for cardiolipin is not higher than for other anionic phospholipids including PS [45,46].

It was recently reported that radiolabeled PS, introduced to rat liver mitochondria by co-incubating with microsomal donor vesicles, appeared to be markedly enriched to contact sites as compared to OM and IM proper [6,7]. In contrast, similar enrichment of the PE product to the contact sites was not observed. In the present study we determined the E/M ratio as a function of Pyr_xPS and (exogenous) Pyr_xPE concentration in hydroxylamine-treated mitochondria. There was no significant differences in the slopes of E/M vs. Pyr_xPS or Pyr_xPE concentration plots independent whether lipids with a short or long pyrenyl acyl was used (Fig. 5), strongly suggesting that Pyr_xPS and Pyr_xPE are similarly laterally distributed in OM. Thus,

if PS is indeed enriched to the contact sites, PE should be similarly enriched. Although this appears to contradict the data obtained by Ardail et al. [6,7], it should be noted that these authors compared the distributions of loaded PS and PE formed thereof while in our case both PS and PE were exogenous. It is possible that endogenous and exogenous PE are differently distributed (laterally and/or transversally) in OM. On the other hand, the facile movement of Pyr_xPE (and -PS) across OM (see below) indicates that exogenous PE mixes rapidly with the total OM PE pool.

In the present study Pyr_{10}PS and its decarboxylation product Pyr_{10}PE were found to be confined to OM independent of the time (0–60 min) and temperature (10–37°C) of incubation. This is in full agreement with the results obtained recently with radiolabeled lipids [9]. Also, in yeast mitochondria, the PS-derived PE was mostly present in OM [36]. Model A could account for this fact if one assumes that the regions involved in the decarboxylation occupy only a small fraction of the total area of IM and, thus, a correspondingly small fraction of PS and PE would be present in IM at any time. The (suggested) annular shape of the semi-fusion sites and absence of transbilayer movement of PS and PE in IM would prevent the access of these phospholipids to IM proper.

The back-exchange experiments (Fig. 4) indicated that a major fraction of both the loaded PS and the PE formed thereof were accessible to the transfer protein, i.e., were exposed to the outer surface of OM. Since it is highly unlikely that the decarboxylase, located exclusively in IM [9,47,48] with its active site exposed to the intermembrane space [49], could act on PS located in the outer leaflet of OM, both PS and PE must be able to move rapidly across OM. This movement could be spontaneous or mediated by a translocase similar to the one found of the plasma membrane [50,51]. However, no evidence for the presence of an aminophospholipid translocase in OM of mammalian mitochondria has been provided and attempts to find such an enzyme in yeast mitochondria have failed [52]. Thus, it is likely that both PS and PE diffuse spontaneously across OM. Because spontaneous diffusion of phospholipid molecules across lipid membranes is generally a slow process due the limited solubility of the polar head group in the apolar membrane core [53], special structures allowing facile transbilayer diffusion of the polar head group of PS and PE should be present in OM. These structures could be equivalent to the pores allowing small molecules (< 5 kDa) pass through OM [54].

As shown above (Fig. 6) the rate of decarboxylation of loaded PS in intact mitochondria was largely independent of lipid hydrophobicity (i.e., the acyl chain length). Assuming that the rate of decarboxylation is not rate limiting (see below), this result suggests that the movement of PS from outer surface of OM to the site of decarboxylation takes place by lateral diffusion, which is only weakly dependent on lipid acyl chain length [35]. Model A is compatible with this result.

According to Model B transport of PS from the inner leaflet of OM to the outer leaflet on IM takes place by spontaneous or protein mediated diffusion through the intermembrane space. Both of these mechanisms are markedly dependent on the hydrophobicity of the the lipid substrate [12,24,32,34] and, thus, the finding that Pyr_xPS decarboxylation (i.e., intramitochondrial transport) rate was largely indenpendent of the lipid acyl chain length (Fig. 6), is not compatible with this model. Previously, attempts to detect (soluble) PS and PE transport activities in the intermembrane space have failed [9]. Model B also cannot readily explain the absence of the PE product from IM.

Model C, previously considered [9,55], assumes that the decarboxylase can act on the PS substrate remaining in the outer membrane. The feasibility of such a model is supported by the fact that the average separation between OM and IM is only 20–80 Å [56]. This model could also readily explain why the PE formed from PS does not mix with the total mitochondrial PE pool [3]. Furthermore, it is compatible with the present data including the independence of the rate of decarboxylation on Pyr_xPS chain length and the absence of the Pyr_{10}PE product from IM.

We conclude that both Models A and C can account for the data obtained so far on intramitochondrial phospholipid transport. While there are more data supporting Model A, it seems premature to discount the Model C yet. Model B, on the other hand, is contradicted by several findings discussed above, and thus seems unlikely.

Usefulness of pyrenyl lipids in studies on mitochondrial lipid transport

The pyrenyl derivatives appear to be very useful tools for studies on mitochondrial lipid transport and distribution. First, their transport to and from mitochondria can be monitored continuously (Fig. 2). Second, discrimination between true insertion of lipid monomers to the target membrane and mere adherence of the donor vesicles can be readily done based on the spectroscopic features (dequenching). Third, the decarboxylation of PS can be determined conveniently and with a high sensitivity by using the HPLC assay. Fourth, these derivatives offer an unique possibility to study lateral distribution of their parent lipids in the mitochondrial membranes by the determination of the E/M ratio as a function of probe concentration (Fig. 5).

Crucial for many of such studies is, however, that the pyrenyl derivatives behave similarly to their natural counterparts. That this is the case is suggested by the

efficient decarboxylation of Pyr_xPS species both in micelles and in intact mitochondria (Figs. 2 and 6), as well as by the fact the distributions of $Pyr_{10}PS$ and -PE between the OM and IM were virtually identical to those found previously for the corresponding natural species [9]. The efficient transport of pyrenyl lipids by intracellular transfer proteins [12,34,57], as well as the facile incorporation of pyrenyl fatty acids to the lipids of cultured cells [33] also support the notion that the pyrenyl derivatives mimic closely their natural counterparts.

The efficient decarboxylation of the Pyr_xPS species also allows one to use these derivatives to investigate PS transport from other organelles to mitochondria in living cells. Pyr_xPS species could be introduced to the cells by incubation with labeled lipid vesicles [26,58] or by metabolic labeling with a pyrenyl fatty acid [33].

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