The fusogenic lipid phosphatidic acid promotes the biogenesis of mitochondrial outer membrane protein Ugo1

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Import and assembly of mitochondrial proteins depend on a complex interplay of proteinaceous translocation machineries. The role of lipids in this process has been studied only marginally and so far no direct role for a specific lipid in mitochondrial protein biogenesis has been shown. Here we analyzed a potential role of phosphatidic acid (PA) in biogenesis of mitochondrial proteins in Saccharomyces cerevisiae. In vivo remodeling of the mitochondrial lipid composition by lithocholic acid treatment or by ablation of the lipid transport protein Ups1, both leading to an increase of mitochondrial PA levels, specifically stimulated the biogenesis of the outer membrane protein Ugo1, a component of the mitochondrial fusion machinery. We reconstituted the import and assembly pathway of Ugo1 in protein-free liposomes, mimicking the outer membrane phospholipid composition, and found a direct dependency of Ugo1 biogenesis on PA. Thus, PA represents the first lipid that is directly involved in the biogenesis pathway of a mitochondrial membrane protein.

Introduction

Most mitochondrial proteins are encoded in the nucleus and have to be imported as cytosolic precursors. The targeting, import, and assembly of these precursors is mediated by sophisticated proteinaceous machineries. They comprise the central preprotein translocase TOM, the sorting and assembly machinery SAM, and the MIM complex in the outer membrane, the MIA machinery in the intermembrane space, and the TIM22 and TIM23 translocases in the inner membrane (Dolezal et al., 2006; Neupert and Herrmann, 2007; Baker et al., 2007; Chacinska et al., 2009; Endo and Yamano, 2010; Dudek et al., 2013). If and how lipids might be involved in mitochondrial protein biogenesis is still unclear. For the two nonbilayer phospholipids cardiolipin (CL) and phosphatidylethanolamine (PE), a role in regulation of TOM complex stability has been proposed, thereby affecting protein biogenesis rather indirectly (Gebert et al., 2009; Becker et al., 2013; Horvath and Daum, 2013). Similarly, CL appeared to be required for the stability of the TIM23 translocation machinery (Jiang et al., 2000; Kutik et al., 2008; Tamura et al., 2009). In addition, a few precursors destined for the outer membrane were found to be imported independently of the known import machineries or to require only import receptors but not the central translocation pore. Therefore it has been speculated that a distinct lipid composition might contribute to outer membrane protein biogenesis (Otera et al., 2007; Merklinger et al., 2012). In particular, Fis1, a component of the mitochondrial fission machinery, appeared to be dependent on a distinct lipid composition of the outer membrane, and its low ergosterol content was proposed to direct the precursor to the outer membrane while the typical high ergosterol levels in other membranes prevent Fis1 import (Krumpe et al., 2012). However, so far no lipid has been identified that directly promotes biogenesis of a mitochondrial protein.

Here, we analyzed the role of phosphatidic acid (PA), a nonbilayer phospholipid, in mitochondrial protein biogenesis. We screened yeast mitochondrial proteins from strains treated with lithocholic acid (LCA) that induces changes in the glycerol-
phospholipid composition of mitochondrial membranes, in particular increased levels of PA (Beach et al., 2013; Burstein and Titorenko, 2014). We found a specific LCA-dependent increase of the outer membrane fusion machinery component Ugo1. A similar increase was found in upslΔ mitochondria, which also exhibit increased levels of PA (Connerth et al., 2012). Using liposomes that mimic the outer membrane phospholipid composition, we show that Ugo1 assembly strictly depends on PA. The efficiency of Ugo1 assembly in protein-free liposomes directly correlates with the amounts of PA in the membranes.

Results

LCA treatment stimulates Ugo1 biogenesis in vivo and in organello

To identify mitochondrial proteins whose biogenesis might depend on a distinct membrane phospholipid composition, we grew yeast cells in the presence or absence of LCA. This bile acid leads to a remodeling of the glycerophospholipid composition of mitochondrial membranes, in particular to increased levels of PA (Beach et al., 2013; Burstein and Titorenko, 2014). We isolated mitochondria from LCA-treated and nontreated cells and analyzed the levels of various classes of mitochondrial proteins by immunoblotting (Fig. 1 A). We found strongly increased levels of Ugo1, a 58-kD subunit of the mitochondrial fusion machinery component Ugo1. A similar increase was found in upslΔ mitochondria, which also exhibit increased levels of PA (Connerth et al., 2012). Using liposomes that mimic the outer membrane phospholipid composition, we show that Ugo1 assembly strictly depends on PA. The efficiency of Ugo1 assembly in protein-free liposomes directly correlates with the amounts of PA in the membranes.

Figure 1. LCA stimulates Ugo1 biogenesis in vivo and in organello. (A) Immunoblot analysis of mitochondria isolated from wild-type yeast cultures that were incubated in the presence or absence of LCA. (B) Quantification of protein levels of mitochondria from LCA-treated or control yeast cultures (−LCA values were set to 100%). Quantifications represent mean ± SEM (error bars; n ≥ 3). (C) Assembly reaction of radiolabeled Ugo1, Por1, and Aac2 precursors in isolated mitochondria from yeast cultures that were incubated in the presence or absence of LCA. Samples were solubilized in digitonin buffer and separated via blue native electrophoresis. Assembly of precursor in mature complexes was visualized by autoradiography. Δψ, membrane potential. (D) Quantification of assembly reactions from C. Values represent mean ± SEM (error bars; n = 3). (E) Two-dimensional blue native/SDS-PAGE of digitonin-lysed mitochondria from a wild-type yeast strain that was cultured in the absence or presence of LCA. TOM, translocase of the outer membrane (MW ∼ 400 kD); Ugo1, Ugo1 complex (MW ∼ 140 kD).

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Deletion of UPS1 leads to increased levels of Ugo1 in mitochondria

Mitochondrial PA is predominantly imported from the ER and serves as a major source for the synthesis of CL at the inner membrane (Horvath and Daum, 2013; Tatsuta et al., 2014; Zhang et al., 2014). While mediation of PA transfer from the ER to the mitochondrial outer membrane is still unclear, the intermembrane space protein Ups1 (Sesaki et al., 2006) has been recently identified to transport PA between the outer and inner membrane in a complex with Mdm35 (Connerth et al., 2012). We therefore aimed to monitor Ugo1 protein levels in mitochondria to gain further indications of a PA-specific role in Ugo1 biogenesis using an LCA-independent in vivo system.

Deletion of UPS1 leads to a specific increase of Ugo1 protein levels compared with wild type (Fig. 1 C and D). Similarly, the assembly pathway of the inner membrane ADP/ATP carrier was unaffected by LCA (Fig. 1 C and D). We also tested import efficiency of Ugo1 precursor in mitochondria from LCA-treated cultures via SDS-PAGE by carbonate extraction (Fujiki et al., 1982; Becker et al., 2008) or by generation of specific proteolytic fragments (Sesaki and Jensen, 2001; Coonrod et al., 2007; Hoppins et al., 2009; Papic et al., 2011). Both assays revealed stimulation upon LCA treatment similar to the import reaction monitored on blue native PAGE (Fig. S1 C). Binding of the Ugo1 precursor to mitochondria was not affected by LCA treatment (Fig. S1 C). To analyze the levels of endogenous Ugo1 complexes, we performed a two-dimensional blue native/SDS-PAGE and found increased amounts upon LCA treatment (Fig. 1 E). Further membrane protein complexes tested were not affected (Fig. S1 D). Thus, Ugo1 biogenesis appears to depend on a striking way on changes in the mitochondrial phospholipid composition induced by LCA treatment and was therefore further tested for a PA-dependent biogenesis pathway.

Import and assembly of Ugo1 was shown to depend on the import receptor Tom70 and the multifunctional outer membrane protein Mim1 but not the general insertion pore of the TOM complex (Becker et al., 2011; Papic et al., 2011). Deletion of either component reduced the assembly efficiency of Ugo1; however, a considerable amount of Ugo1 dimers were still formed (Becker et al., 2011). We therefore tested if Ugo1 assembly is also possible in the absence of Tom70 and Mim1 and established an Ugo1 assembly assay using protein-free liposomes that mimic the phospholipid composition of the outer membrane. This robust in vitro system would also allow us to directly assess a PA requirement for Ugo1 biogenesis. The amounts of various phospholipids in mitochondrial outer membranes from yeast were determined by two-dimensional thin-layer chromatography (Zinsen et al., 1991; de Kroon et al., 1999). We generated highly pure outer membrane fractions that were largely devoid of inner membrane (Tim23) and ER marker proteins (Sec61, Sss1; Fig. S2 A) and determined the phospholipid composition by mass spectrometry (LC-MS). We found similar values for the various classes of phospholipids as obtained by phospholipid separation through thin-layer chromatography and lipid analyses (Fig. S2 B; Zinsen et al., 1991; de Kroon et al., 1999). We used the phospholipid profile to generate large unilamellar vesicles (OMLs) mimicking the phospholipid composition of the mitochondrial outer membrane (Muir et al., 2003; see Materials and methods). OMLs were incubated with 35S-labeled precursor proteins. Optionally, Proteinase K digestion was used to remove nonimported precursors and outer surface-exposed parts of the proteins. OMLs were resolated using sedimentation ultracentrifugation. To separate OMLs from precursors and potentially aggregated protein species, samples were floated in a sucrose gradient (van der Laan et al., 2007). After resolation, samples were analyzed via SDS-PAGE or blue native electrophoresis followed by autoradiography (Fig. 3 A). In parallel, all precursors were imported into iso-
lated mitochondria. As shown in Fig. 3 B, only Ugo1 precursor revealed a strong binding to OMLs while other outer membrane precursor proteins of various topological classes imported only into mitochondria but not OMLs.

The topology of Ugo1 has been analyzed by treating isolated mitochondria with Proteinase K or Trypsin and can be monitored by the appearance of specific fragments (Sesaki and Jensen, 2001; Coonrod et al., 2007; Hoppins et al., 2009; Papic et al., 2011). To analyze the topology of Ugo1 in OMLs, we compared the proteolytic pattern of imported Ugo1 in isolated mitochondria and OMLs. The generation of Trypsin protected fragments of Ugo1 was directly comparable in OMLs and mitochondria, as two dominant fragments at ∼32 and 22 kD appeared (Fig. 4 A, f1 and f2), indicating a correct topological orientation of Ugo1 upon import in OMLs.

To further analyze membrane integration of Ugo1 into OMLs, we treated mitochondria and OMLs with alkaline pH (Fujiki et al., 1982) after import reactions. An ultracentrifugation step separated pellet (integral membrane proteins) and supernatant (soluble or peripherally attached proteins) fractions. Ugo1 was efficiently recovered in the pellet fraction after import into OMLs and mitochondria but not in mock-treated samples, indicating a stable integration of Ugo1 into the lipid bilayer (Fig. 4 B). Moreover, Ugo1 membrane insertion was dependent on the applied temperature (Fig. S3 A). We conclude that Ugo1 adapts its native transmembrane topology in the protein-free lipid bilayer of OMLs.

We then asked if Ugo1 precursor also forms a dimeric complex after import into OMLs. Therefore, we imported radiolabeled Ugo1 precursor into isolated mitochondria and OMLs and separated the samples after solubilization with 1% (wt/vol) digitonin buffer via blue native electrophoresis. The 140-kD mature form of Ugo1 appeared in OMLs like in mitochondria in a time-dependent manner (Fig. 4 C). Next, we tested if the formation of the Ugo1 membrane assembly is proportional to the amount of lipids present. Indeed, increasing concentration of OMLs led to an increase in Ugo1 assembly correlating Ugo1 import and assembly with the provided surface of OMLs (Figs. 4 D and S3 B). Collectively, Ugo1 precursor imports and integrates into protein-free lipid bilayers, engaging in its correct topological orientation. Furthermore, Ugo1 matures into its native 140-kD dimeric form as shown by blue native electrophoresis, and the efficiency of this assembly correlates with the amount of lipids present.

Assembly of Ugo1 in protein-free liposomes depends on PA
PA is the major up-regulated lipid species in mitochondria upon LCA treatment (Beach et al., 2013; Burstein and Titorenko, 2014) and specifically increased upon deletion of Ups1, the protein that shuttles PA from the outer to the inner membrane for CL synthesis (Connerth et al., 2012). In both in vivo systems, we observed a specific stimulation of Ugo1 biogenesis (Figs. 1 and 2). We therefore wondered if the assembly of Ugo1 in protein-free OMLs might depend on the presence of PA. To address this, we analyzed Ugo1 assembly in PA-containing and PA-free OMLs. Ugo1 precursor was imported into these OMLs and its assembly was analyzed via blue native electrophoresis. Formation of the 140-kD Ugo1 dimer was strongly reduced in the absence of PA (Fig. 5, A and B) and could be stimulated by increasing amounts of PA present in OMLs (Fig. 5 C). Lack of other lipids, like CL, phosphatidylglycerol (PG), or phosphatidylserine (PS), the levels of which were also reported to be changed upon LCA treatment or ups1 deletion (Connerth et al., 2012; Beach et al., 2013; Burstein and Titorenko, 2014), did not affect assembly of Ugo1 in OMLs (Fig. S4). Hence,
PA promotes Ugo1 biogenesis

Vögtle et al.

PA emerges here as a major player that is directly involved in the biogenesis of Ugo1.

**LCA treatment and UPS1 deletion stimulate Ugo1 biogenesis in mim1Δ cells**

Our results indicated that proteinaceous factors, like Mim1, are dispensable for import and assembly of Ugo1 into its dimeric complex. However, this process seems to strictly depend on the phospholipid PA. We therefore wondered if increased PA levels might also stimulate Ugo1 biogenesis in the absence of Mim1 in vivo. We isolated mitochondria from mim1Δ cells, which were treated with LCA as described above (Fig. 1), and analyzed Ugo1 protein levels. We found that LCA treatment led to a similar increase of Ugo1 protein levels in mim1Δ cells, which were treated with LCA as described above (Fig. 1), and analyzed Ugo1 protein levels. We found that LCA treatment led to a similar increase of Ugo1 protein levels in mim1Δ mitochondria (Fig. 6, A and B) as observed previously in wild-type mitochondria (Fig. 1, A and B). Ugo1 assembly is also strongly enhanced upon LCA treatment, as revealed by two-dimensional blue native/SDS-PAGE analysis (Fig. 6 C). A very similar stimulation of Ugo1 biogenesis was observed when ups1 was deleted in the mim1Δ background to increase PA levels in vivo (Fig. 6, D–F; and Fig. S5). These results indicate a predominant role of PA in Ugo1 biogenesis, which functions independently of Mim1.

**Discussion**

Our study reveals two novel insights into the biogenesis pathway of Ugo1. (1) Import and assembly of Ugo1 precursors can function in the absence of proteinaceous factors. Thus, no other outer membrane proteins are strictly required for Ugo1 biogenesis. The reported dependence of Ugo1 biogenesis on the import receptor Tom70 and Mim1 (Becker et al., 2011; Papic et al., 2011) might represent a mechanism that accelerates this process under in vivo conditions. (2) Ugo1 biogenesis strictly depends on the fusogenic phospholipid PA in a reconstituted system using protein-free liposomes, and the efficiency of Ugo1 import and assembly in organello and in vivo positively correlates with PA levels in mitochondria. With a concentration of
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Figure 5. Assembly of Ugo1 requires PA. (A) \(^{35}\)S-labeled Ugo1 was imported into isolated mitochondria (Mito.) and OMLs lacking or containing PA (0.4%). Samples were solubilized in digitonin buffer and separated by blue native electrophoresis and analyzed by digital autoradiography. (B) Quantification of the 140 kD assembly of Ugo1 in OMLs analyzed as described in A. Values were adjusted to the lipid amount of the samples analyzed and set to 100% for OMLs containing PA. Quantifications represent mean ± SEM (error bars; n = 3). (C) Radiolabeled Ugo1 was imported into mitochondria (Mito.) or OMLs with increasing concentrations of PA. Samples were solubilized in digitonin buffer and analyzed by blue native electrophoresis and digital autoradiography.

~0.4 mol%, PA is present in only small amounts in the outer membrane (Fig. S2 B). However, a slight reduction of this concentration in OMLs is sufficient to dramatically decrease Ugo1 assembly efficiency, reflecting a direct dependency on this lipid (Fig. 5). While CL and PE were shown to influence mitochondrial protein biogenesis indirectly by stabilizing structure and function of preprotein translocases (Jiang et al., 2000; Kutik et al., 2008; Gebert et al., 2009; Tamura et al., 2009; Becker et al., 2013), our results uncovered PA as the first lipid that directly promotes biogenesis of a mitochondrial membrane protein.

PA was the major up-regulated lipid species upon LCA treatment (Beach et al., 2013; Burstein and Titorenko, 2014) and has been shown to facilitate mitochondrial fusion in mammals by a so-far unknown mechanism (Choi et al., 2006; Yang and Frohman, 2012). Notably, LCA treatment of yeast also leads to increased mitochondrial fusion activity that is likely caused by increased PA level (Beach et al., 2013; Burstein and Titorenko, 2014). It has been proposed that PA, which possesses a small, negatively charged head group, influences the negative curvature of mitochondrial membranes (Yang and Frohman, 2012; Zhang et al., 2014), thereby promoting membrane fusion. Given the very low content in the outer mitochondrial membrane, it is difficult to envision how PA may control the overall phospholipid organization, leading to membrane bending (Fig. S2 B; Zinser et al., 1991; de Kroon et al., 1999; Mejia and Hatch, 2015). It is therefore tempting to speculate that PA might exert its fusogenic potential by supporting assembly of Ugo1 that is required in yeast to activate conserved GTPases/mitofusins, mediating membrane fusion (Wong et al., 2003; Sesaki and Jensen, 2004; Anton et al., 2011). (A mammalian Ugo1 homologue has not yet been found.) A possible role of Mim1 in such a scenario might be to concentrate PA into foci to stimulate Ugo1 biogenesis at the outer membrane in vivo. Additionally, proteinaceous factors like Mim1 and the import receptor Tom70 may play a role in the targeting of Ugo1 precursor to mitochondria in vivo. Due to the presence of PA in other cellular compartments, a specific mitochondrial recognition mechanism for the Ugo1 precursor will be required. In line with this, our results revealed that in contrast to membrane insertion and assembly, the mitochondrial targeting of Ugo1 precursor does indeed not depend on PA (Fig. S1 C). However, the subsequent steps in Ugo1 biogenesis including membrane insertion and assembly into homodimetic complexes occur in a protein-independent manner and require the phospholipid PA.

Materials and methods

Yeast strains and growth conditions

The following yeast strains were used in this study: YPH499 (MATa, ade2-101, his3-D200, leu2-Δ1, ura3-52, trp1-Δ63, lys2-801), BY4741 (MATa, his3-DΔ1, leu2Δ0, met15Δ0, ura3Δ0), and mim1Δ (MATa, ade2-101, his3-D200, leu2-Δ1, ura3-52, trp1-Δ63, lys2-801, mim1::ADE2; Becker et al., 2008). Ugo1 was chromosomally tagged with a C-terminal 3HA tag using as a selectable marker the His3MX6 module (MATa, ade2-101, his3-D200, leu2-Δ1, ura3-52, trp1-Δ63, lys2-801, ugo1::UGO1HA3-HIS3; Longtine et al., 1998). UPS1 was deleted in the mim1Δ background by homologous recombination using a HIS3 cassette (MATa, ade2-101, his3-D200, leu2-Δ1, ura3-52, trp1-Δ63, lys2-801, mim1::ADE2, ups1::HIS3; Longtine et al., 1998). Yeast cells were grown on YPG medium (1% [wt/vol] yeast extract, 2% [wt/vol] bacto-peptone, and 3% [wt/vol] glycerol) at 21°C to an OD of 1.5. For LCA treatment, yeast cells were grown to mid-logarithmic growth phase in medium containing 0.2% [wt/vol] glucose, 1% [wt/vol] yeast extract, and 2% [wt/vol] bacto-peptone, and supplemented with 50 µM LCA in DMSO (final concentration of DMSO, 1% [vol/vol]). Non-treated cells received DMSO only. Cells were harvested after growth at 30°C for 48 h (Beach et al., 2013).

Isolation of highly purified mitochondrial outer membrane vesicles (OMVs)

Wild-type yeast cells (YPH499) were grown on YPG medium at 24°C to an OD of 1.5. Cells were harvested and mitochondria were isolated using differential centrifugation (Meisinger et al., 2000). Highly purified mitochondria were obtained by further sucrose gradient purification (Meisinger et al., 2000). For isolation of OMVs, 50 mg of highly purified mitochondria were resuspended in swelling buffer containing 5 mM potassium phosphate, pH 7.5, and 1 mM phenylmethylsulfonyl fluoride (4 mg/ml protein concentration) and incubated for 20 min on ice. The sample was treated with a Teflon glass potter with 20 strokes.
and loaded on a three-step sucrose gradient (1 ml 60%, 4 ml 32%, and 1 ml 15% sucrose (wt/vol) in EM buffer (10 mM MOPS/KOH, pH 7.2, and 1 mM EDTA). Centrifugation was performed in a SW41 swing-out rotor for 1 h at 134,000 g). OMVs were collected from the 15–32% interface and after adjustment of sucrose concentration to 50% (wt/vol), floated (from the bottom) on a sucrose gradient (5 ml 32% (wt/vol)).
vol] sucrose in EM buffer and 1.5 ml EM buffer) at 240,000 g overnight. Highly pure OMVs were obtained from the 0–32% interface and resuspended after pelleting in EM buffer (Zahedi et al., 2006).

**Lipid analysis**

For lipid profiling by mass spectrometry, highly purified mitochondria and outer membrane vesicles were extracted using the Bligh-Dyer method (Bligh and Dyer, 1959). Di-C12:0 PE, di-C10:0 PG, di-C10:0 PS, tetra-C14:0 CL, C12:0-C13:0 PA (Avanti Polar Lipids), and di-C8:0 GPhns (Echelon Biosciences) were added as internal lipid standards. Quantification of individual molecular species was performed using multiple reaction monitoring (MRM) with an Applied Biosystems 4000 Q-Trap mass spectrometer (Applied Biosystems), with upfront normal phase liquid chromatography separation (Agilent 1100 LC), as described previously (Guan et al., 2010; Shui et al., 2010). Each lipid species (defined by the sum of carbons and double bonds of the acyl chains) were quantified using the parent >headgroup fragment transitions. Each individual ion dissociation pathway was optimized with regard to collision energy and fragmentor voltage to minimize variations in relative ion abundance due to differences in dissociations. Lipid levels were normalized to the internal standards and calculated as mol% of total lipid measured. For analysis of the lipid composition by two-dimensional thin-layer chromatography, lipids were isolated from 1 mg mitochondria with chloroform/methanol (2:1 [vol/vol]; Folch et al., 1957). Non-lipid contaminants were removed from the extracts by several washing steps including a 0.035% (wt/vol) MgCl₂ solution, 2 M KCl/methanol (4:1 [vol/vol]), and methanol/water/chloroform (48:47:5 [vol/vol/vol]). Lipid extracts were applied to thin-layer chromatography plates (Silica gel 60; Merck) and developed in an ascending manner using the solvent systems chloroform/methanol/25% NH₃ (68:35:5 [vol/vol/vol]) in the first dimension (Schneiter and Daum, 2006). Individual phospholipids were stained with 0.8 g MnCl₂ (w/v; Lotan and Schachter, 1972) and visualized after charring at 100°C for 30 min.

**In organello protein import**

Radiolabeled precursor proteins were synthesized in the presence of [³⁵S]methionine (PerkinElmer) in rabbit reticulocyte lysate (Promega). For in organello import assays, 85S precursors were incubated with 30 µg of isolated yeast mitochondria in BSA buffer (10 mM MOPS/KOH, pH 7.2, 3% [wt/vol] BSA, 250 mM sucrose, 5 mM MgCl₂, 80 mM KCl, and 5 mM KP) and supplemented with 2 mM ATP and 2 mM NADH. Samples were incubated for the indicated time periods at 25°C. Reactions were stopped on ice and treated with 50 µg/ml Proteinase K for 10 min where indicated. After addition of 2 mM PMSF (phenylmethylsulfonyl fluoride in isopropanol) and additional incubation for 10 min, samples were centrifuged at 16,000 g for 10 min at 4°C. Samples were suspended in 200 µl SEM buffer (250 mM sucrose, 1 mM EDTA, and 10 mM MOPS/KOH, pH 7.2) and centrifuged as before. Samples were analyzed by SDS-PAGE or blue native electrophoresis followed by autoradiography and immunodecoration (Table S1).

**Blue native electrophoresis**

50 µg mitochondria or 150–250 µg OMLs were solubilized in 1% (vol/vol) digitonin solubilization buffer after import reaction with radiolabeled precursor protein was completed (Becker et al., 2011). Samples were centrifuged at 16,000 g for 10 min at 4°C, and supernatant was loaded on a 4–16.5% blue native gradient gel followed by autoradiography. For two-dimensional blue native/SDS-PAGE, gel lanes from 1D blue native electrophoresis were cut and polymerized on top of a 12.5% SDS-polyacrylamide gel.

**Generation of large unilamellar vesicles mimicking the outer membrane lipid composition (OMLs)**

Lipids dissolved in a mixture of chloroform/methanol (9:1 [vol/vol]) were mixed in order to obtain a final lipid composition as determined by the analysis of the outer mitochondrial membrane by liquid chromatography–mass spectrometry (LC-MS; Fig. S2): PC (37.9%), CL (1.6%), PA (0.4%), PE (28.9%), PG (1.1%), phosphatidylinositol (PI, 29.8%), and PS (0.1%). Lipids were purchased from Sigma-Aldrich, except for PC, which was provided by Lipoid. Values are given in percentage of weight. The lipid mixture was labeled with 0.2% (wt/wt) rhodamine PE (Molecular Probes) and dried under a vacuum. By addition of buffer (135 mM KCl, 10 mM Hepes, and 1 mM MgCl₂, pH 7.0) to the lipid film, a crude liposomal suspension with a lipid concentration of 10 mg/ml was obtained. By extruding the suspension 51 times with a LiposFast extruder (Avestin) through a nucelpore polycarbonate track-etched membrane (Whatman; GE Healthcare) of 100 nm pore size, a suspension of unilamellar vesicles with a narrow size distribution was prepared. Vesicles of a diameter <30 nm were removed by centrifugation at 288,000 g for 60 min. Thus, only vesicles with a diameter of ~100 nm were used in further studies. Quality control measurements to confirm size and unilamellarity were performed by means of dynamic light scattering (mean hydrodynamic radius [Z average]: 113 nm ± 9.2 nm; mean polydispersity index: 0.0627 ± 0.013). In addition, quality was controlled by cryotransmission electron microscopy as described previously (Holzer et al., 2009). In brief, 3 µl of the diluted liposome dispersion (5–10 mM) were applied on a 400 × 100 mesh Quantifoil S7/2 holey carbon film on copper grids (Quantifoil Micro Tools GmbH). Excess liquid was removed from the grid with filter paper, and the sample was immediately shock-frozen by injection into liquid ethane. Sample preparation steps were performed in a climate-controlled room using a CryoBox 340719 (Carl Zeiss). The subsequent fixation of the grid on the sample rod (626-DH; Gatan, Inc.) and transfer of the rod into the transmission electron microscope (Leo 912 Omega; Leo) were performed under a nitrogen atmosphere at a temperature of 90 K (~183°C). The instrument was operated at 120 kV. Finally, the total lipid concentration was measured using the Bartlett method for phosphate quantification (Bartlett, 1959).

**Protein import in OMLs**

Radiolabeled precursors were mixed with 150–250 µg OMLs in reaction buffer (3 mM NaN₃, 2 mM KH₂PO₄, and 50 mM NaCl, pH 7.0). Samples were incubated at 25°C (or 4°C as controls) for 20–40 min and reactions were stopped by placing the samples on ice. Non-imported precursors were removed by Proteinase K digestion (50 µg/ml) for 10 min on ice. Digestion was stopped by the addition of 2 mM PMSF, and OMLs were resolubilized by centrifugation at 288,000 g for 45 min at 4°C. OMLs were washed in 400 µl of reaction buffer and centrifuged. The obtained pellet was resuspended in 150 µl of 1.4 M sucrose and overlaid with 400 µl of 1 M sucrose. The sucrose gradient was overlaid with 200 µl of reaction buffer. Samples were centrifuged as before and OMLs were recovered in the interface between 1 M sucrose and reaction buffer. OMLs were washed in reaction buffer to remove residual sucrose and dissolved in Laemmli buffer for analysis on SDS-PAGE or in 1% (vol/vol) digitonin buffer for blue native electrophoresis (van der Laan et al., 2007). For separation of integral and peripheral or soluble proteins, mitochondria or OMLs were resolubilized after import and suspended in 400 µl of 100 mM Na₂CO₃. Samples were mixed and incubated on ice for 30 min. Pellet and supernatant fractions were
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