Phosphatidylethanolamine and Cardiolipin Differentially Affect the Stability of Mitochondrial Respiratory Chain Supercomplexes

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The mitochondrial inner membrane contains two non-bilayer-forming phospholipids, phosphatidylethanolamine (PE) and cardiolipin (CL). Lack of CL leads to destabilization of respiratory chain supercomplexes, a reduced activity of cytochrome c oxidase, and a reduced inner membrane potential Δψ. Although PE is more abundant than CL in the mitochondrial inner membrane, its role in biogenesis and assembly of inner membrane complexes is unknown. We report that similar to the lack of CL, PE depletion resulted in a decrease of Δψ and thus in an impaired import of preproteins into and across the inner membrane. The respiratory capacity and in particular the activity of cytochrome c oxidase were impaired in PE-depleted mitochondria, leading to the decrease of Δψ. In contrast to depletion of CL, depletion of PE did not destabilize respiratory chain supercomplexes but favored the formation of larger supercomplexes (megacomplexes) between the cytochrome bc₁ complex and the cytochrome c oxidase. We conclude that both PE and CL are required for a full activity of the mitochondrial respiratory chain and the efficient generation of the inner membrane potential. The mechanisms, however, are different since these non-bilayer-forming phospholipids exert opposite effects on the stability of respiratory chain supercomplexes.

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and essential for cell survival, \(^2,4\) whereas CL is specific for mitochondria. \(^2,4,8\) The synthesis of CL takes place in the inner mitochondrial membrane, where the CL synthase (CRD1) catalyzes the formation of CL from phosphatidylglycerol and CDP-diacylglycerol. \(^2,10\) In yeast, the majority of PE is generated by deacylation of phosphatidylinerine catalyzed by phosphatidylinerine deacylases (Psd). Two Psd enzymes have been described. Psd1 plays the major role in PE synthesis and is located in the mitochondrial inner membrane/intermembrane space. \(^2\) Smaller amounts of PE are generated by the Psd2 activity of the Golgi apparatus/vacuole membrane, as well as by the CDP-ethanolamine pathway and the acyltransferases Tgl3 and Ale1. \(^1,4,13,17\)

PE binds to respiratory chain complexes \(^43,64\) and in vivo data indicate an important role of PE for mitochondrial functions. \(^6,29\) Studies with lactose permease revealed a role of PE in folding and activity of membrane proteins in Escherichia coli. \(^65\) The effect of PE depletion on mitochondrial processes, however, has not been studied on a molecular level.

Here, we report that protein transport into and across the inner membrane is impaired in PE-depleted mitochondria. The protein translocases and inner membrane complexes are not dissociated upon lack of PE, but the activity of the respiratory chain, in particular of cytochrome c oxidase, is impaired, leading to a reduction of Δψ. Thus, the reduced Δψ leads to an impairment of protein import into the inner membrane. In contrast to the lack of CL, lack of PE stabilizes supercomplexes of the respiratory chain and does not block the formation of AAC oligomers. Though both PE and CL are required for respiratory activity and efficient generation of a Δψ by mitochondria, they play opposing roles in the stabilization of protein complexes.

**PE-depleted mitochondria are impaired in preprotein transport to the inner membrane**

To study the role of PE in mitochondrial protein biogenesis, we used a Saccharomyces cerevisiae strain lacking Psd1 and a double deletion strain lacking Psd1 and Psd2. \(^69\) Both yeast strains exhibited a poor growth on non-fermentable carbon sources and were sensitive to growth at high and low temperatures (Fig. S1a). For further analysis, we grew the cells at an intermediate temperature (30 °C, early logarithmic growth phase) on non-fermentable carbon sources and analyzed the phospholipid profiles of cell extracts from psd1Δ and psd1Δ psd2Δ strains. \(^6\) The level of PE (29 mol% of total phospholipids in wild-type cells) was considerably decreased in psd1Δ cells (7%) and strongly reduced in the double deletion mutant (1%) (Fig. S1b). \(^6\) We also determined the content of total phospholipids in purified inner membrane vesicles from these mutants and observed a moderate reduction of the phospholipid-to-protein ratio compared to wild type (Fig. S1c). Moreover, we determined the
absolute amounts of individual phospholipid classes in inner membrane vesicles from wild-type, \textit{psd1} Δ, and \textit{psd1} Δ \textit{psd2} Δ mitochondria. \begin{figure}
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\includegraphics[width=\textwidth]{fig1.png}
\caption{PE is selectively depleted in inner membrane vesicles from \textit{psd1} Δ and \textit{psd1} Δ \textit{psd2} Δ mitochondria. The \textit{S. cerevisiae} strains \textit{crd1} Δ, \textit{psd1} Δ, and \textit{psd1} Δ \textit{psd2} Δ in the BY4741 background \cite{49} were grown in YPLac medium \cite{49} at 30°C to early logarithmic growth phase. Mitochondria were isolated by differential centrifugation, the protein concentrations were adjusted, and inner membrane vesicles were isolated by sucrose gradient centrifugation as previously described. \cite{49,70} Phospholipids were extracted, separated by thin-layer chromatography, and analyzed as reported previously. \cite{69,71,72} The amounts of individual phospholipid classes were determined using a phosphate solution with 1 mg/ml phosphor as standard. \cite{73} Shown are the mean values of two determinations with range. DMPE, dimethylphosphatidylethanolamine; LP, lysophospholipids; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.}
\end{figure}

For protein import experiments, mitochondrial precursor proteins were synthesized in reticulocyte lysates and labeled with [\textsuperscript{35}S]methionine. We used three presequence-containing preproteins and incubated them with mitochondria isolated from \textit{psd1} Δ and \textit{psd1} Δ \textit{psd2} Δ strains: cytochrome \textit{c}_1 is inserted into the inner membrane, whereas subunit \textit{β} of the F\textsubscript{1}F\textsubscript{0}-ATP synthase (F\textsubscript{1}\textit{β}) and the model preprotein Su9-DHFR are translocated across the inner membrane into the matrix. \cite{61,74,77} Each of these preproteins was imported in a Δ\textit{ψ}-dependent manner and the presequences were proteolytically removed (Fig. 2a). Import of the three preproteins was reduced in \textit{psd1} Δ mitochondria and strongly reduced in \textit{psd1} Δ \textit{psd2} Δ mitochondria (Fig. 2a, lanes 5–7 and 9–11). The import of AAC via the carrier pathway was analyzed by monitoring assembly of AAC in the inner membrane. \cite{80,81} We imported AAC into isolated mitochondria, lysed the mitochondria with the non-ionic detergent digitonin, and studied AAC assembly by blue native electrophoresis (Fig. 2b). The biogenesis of AAC was moderately reduced in \textit{psd1} Δ and \textit{psd1} Δ \textit{psd2} Δ mitochondria (Fig. 2b, lanes 5–7 and 9–11).

It has been reported that in CL-deficient mitochondria, the stability of the TIM23 translocase is partially affected. \cite{39,58,60} We thus tested whether depletion of PE also affected the stability of the TIM23 or TIM22 complexes. The protein levels of subunits of the TIM23 complex (Tim17, Tim23) and the TIM22 complex (Tim22, Tim54) were comparable in \textit{psd} mutant and wild-type mitochondria (Fig. S2). The stability of the translocases was analyzed by blue native electrophoresis of digitonin-lysed mitochondria (Fig. 2c). The TIM23 translocase forms several blue native-stable complexes, \cite{39,58,77} which were not dissociated but only slightly shifted to faster migrating forms in \textit{psd1} Δ and \textit{psd1} Δ \textit{psd2} Δ mitochondria (Fig. 2c, lanes 2 and 3) (the slight mobility shifts may indicate that PE is bound to TIM23 in wild-type mitochondria but not critical for the stability of the translocase). The mobility of the TIM22 translocase was not affected in the mutant mitochondria (Fig. 2c, lanes 5 and 6). One hallmark of mitochondria lacking CL is the dissociation of AAC oligomers. \cite{35,39} In contrast, depletion of PE did not block the oligomerization of AAC but only led to a slight mobility shift of AAC oligomers (Fig. 2c, lanes 8 and 9).

In summary, the import of presequence-carrying preproteins and carrier proteins is impaired in PE-depleted mitochondria. The main import machineries TIM23 and TIM22, as well as the AAC oligomers,
are not dissociated when PE is depleted, indicating that PE is not crucial for the stability of these complexes.

**PE is required for the activity of the respiratory chain**

We noticed that the mitochondrial import of the precursor of F1β was more severely affected by PE depletion than the import of Su9-DHFR (Fig. 2a). It was previously shown that the import of F1β requires a higher membrane potential and is thus more sensitive to a reduction of \( \Delta \psi \) than the import of Su9-DHFR, raising the possibility that the preprotein import defects observed in PE-depleted mitochondria may be related to a reduction of \( \Delta \psi \) in the mutant mitochondria.

To assess the membrane potential of mitochondria, we used a \( \Delta \psi \)-sensitive fluorescent dye. PE-depleted mitochondria are impaired in import of preproteins into and across the inner membrane. (a) Isolated mitochondria from wild-type, psd1Δ, and psd1Δ psd2Δ yeast strains were incubated with the \(^{35}\text{S}\)-labeled precursors of cytochrome c (Cyt1), F1β, and Su9-DHFR in import buffer [3% (w/v) bovine serum albumin, 250mM sucrose, 80mM KCl, 5mM MgCl₂, 5mM methionine, 2mM KH₂PO₄, 10mM Mops/KOH, pH7.2, 2mM creatine phosphate, 0.1mg/ml creatine kinase, and 2mM ATP] at 25°C for the indicated periods. In control reactions, the membrane potential (\( \Delta \psi \)) was dissipated prior to import by addition of 8\( \mu \)M antimycin A, 1\( \mu \)M valinomycin, and 20\( \mu \)M oligomycin. The import reactions were stopped by adding 8\( \mu \)M antimycin A, 1\( \mu \)M valinomycin, and 20\( \mu \)M oligomycin. After washing with SEM buffer (250mM sucrose, 1mM ethylenediaminetetraacetic acid, and 10mM Mops/KOH, pH7.2), the mitochondria were lysed under denaturing conditions and subjected to SDS-PAGE followed by digital autoradiography. p, precursor; i, intermediate; m, mature. (b) Isolated mitochondria from wild-type, psd1Δ, and psd1Δ psd2Δ yeast strains were incubated with \(^{35}\text{S}\)-labeled AAC at 25°C as indicated in the presence or absence of \( \Delta \psi \). The mitochondria were lysed with 1% (w/v) digitonin in digitonin buffer [20mM Tris/HCl, pH7.4, 50mM NaCl, 0.1mM ethylenediaminetetraacetic acid, and 10% (v/v) glycerol] and protein complexes were separated by blue native electrophoresis. \(^{35}\text{S}\)-labeled proteins were detected by digital autoradiography. (c) Wild-type, psd1Δ, and psd1Δ psd2Δ mitochondria were lysed with 1% (w/v) digitonin in digitonin buffer and subjected to blue native electrophoresis. Protein complexes were detected by Western blotting using the indicated antisera.
PE and Mitochondrial Respiratory Chain

Fig. 3. PE is required for the activity of the respiratory chain. (a) The membrane potential (Δψ) of wild-type, psd1Δ, and psd1Δ psd2Δ yeast mitochondria was assessed at 25°C by fluorescence quenching using the Δψ-sensitive dye DSC3(5) (3,3′-dipropylthiadicarbocyanine iodide) in membrane potential buffer [0.6 M sorbitol, 0.1% (w/v) bovine serum albumin, 10mM MgCl2, 0.5mM ethylenediaminetetraacetic acid, and 20mM KPi, pH7.4] as described previously.39,82 (b) The oxygen consumption of isolated wild-type, psd1Δ, and psd1Δ psd2Δ mitochondria was analyzed by oxygraph measurements at 25°C. Isolated yeast mitochondria (100μg protein) were added to 2 ml of buffer (10mM Mops/KOH, pH7.4, 250mM sucrose, 5mM MgCl2, 80mM KCl, 5mM KPi, 1mM ADP, and 1mM NADH) and oxygen consumption was measured. The oxygen flux (negative time derivative of oxygen concentration) corrected for instrumental background flux was expressed in picomoles per second per millilitre. Shown are the mean values with standard error of the mean (n=3). (c and d) The activity of the cytochrome bc1 complex (c) and the cytochrome c oxidase (d) was determined in submitochondrial particles prepared from wild-type, psd1Δ, and psd1Δ psd2Δ yeast cells as described earlier.83,84 Ubiquinol-dependent cytochrome c reduction was measured as described by Palsdottir and Hunte using 3μg protein (submitochondrial particles), 50μM horse heart cytochrome c, and 80μM decylubiquinol for 1 ml assay volume (40mM potassium phosphate buffer, pH7.4, 1mM NaN3, and 0.05% β-D-undecylmaltoside). Reduction of cytochrome c was monitored at 550nm and the activity was calculated with an extinction coefficient of 19.4mM⁻¹ cm⁻¹⁻¹. The activity was fully sensitive to the specific inhibitor stigmatellin (1μM). Cytochrome c oxidase activity was measured as described by Horvath et al.,30 with 50μM reduced horse heart cytochrome c and 3–50μg of protein (submitochondrial particles) in 1 ml assay volume (75mM potassium phosphate buffer, pH7.4, 1mM antimycin A, and 0.05% β-D-dodecylmaltoside). Oxidation of cytochrome c was monitored and quantified as for the cytochrome bc1 complex. The activity was fully sensitive to the specific inhibitor sodium azide (1μM). Specific enzyme activities are based on total protein determined by bichinonic acid assay (Pierce). Three preparations per strain were used and the activity measurements were repeated five times for each sample. Mean values with standard error of the mean are shown. (e) The activity of the mitochondrial ATPase was assessed by in-gel calcium phosphate precipitation upon ATP hydrolysis.86,87 Mitochondria isolated from wild-type, psd1Δ, or psd1Δ psd2Δ strains were lysed with 1% (w/v) digitonin in digitonin buffer and protein complexes were separated by blue native electrophoresis. Subsequently, the gel was washed with water and incubated with ATP-containing buffer (50mM glycine, pH8.4, 5mM MgCl2, and 20mM ATP) for 20min and transferred into 10% (w/v) CaCl2 solution. Incubation was performed until calcium phosphate precipitation became visible and the reaction was stopped by transfer into water. V2, ATP synthase dimer; V, ATP synthase monomer; F1, F1 part of the ATP synthase.

Reduced in psd1Δ mitochondria and more severely decreased in psd1Δ psd2Δ mitochondria (Fig. 3b). Budding yeast does not contain complex I of the respiratory chain. The cytochrome bc1 complex and cytochrome c oxidase are the two proton-pumping respiratory complexes and thus we analyzed their activities individually. The activity of the cytochrome bc1 complex was only marginally affected.
by the depletion of PE (Fig. 3c), whereas the activity of the cytochrome c oxidase was considerably reduced (Fig. 3d). The protein levels of several subunits of cytochrome c oxidase such as Cox1 were moderately reduced in the psd1 mutant mitochondria (Fig. S2), supporting the conclusion that cytochrome c oxidase was affected in the mutants. The F1Fo-ATP synthase was visualized in native gels by ATPase activity staining, revealing comparable activities in wild-type and psd mutant mitochondria (Fig. 3e).

Taken together, the stepwise decrease of PE levels in psd1Δ and psd1Δ psd2Δ mutants correlates with the stepwise decrease of cytochrome c oxidase activity, oxygen consumption, membrane potential, and preprotein import into and across the inner membrane. Thus, both PE and CL are important for the activity of the respiratory chain and the generation of a membrane potential. A decreased Δψ results in an impaired protein import into and across the inner membrane.74,76

Lack of PE stabilizes respiratory chain supercomplexes

The cytochrome bc1 complex and cytochrome c oxidase form supercomplexes that can be resolved by blue native electrophoresis. The supercomplexes contain a dimer of the cytochrome bc1 complex (III2) and one (III2/IV) or two copies (III2/IV2) of cytochrome c oxidase (Fig. 4a and b).44,45 We lysed mitochondria with digitonin and analyzed the protein complexes by blue native electrophoresis and immunodecoration. Surprisingly, the supercomplexes were not dissociated in psd1Δ and psd1Δ psd2Δ mitochondria, but rather formed a larger oligomeric assembly, containing both cytochrome bc1 complex and cytochrome c oxidase (Fig. 4a, lanes 2 and 3; Fig. 4b, lanes 2, 3, 5, and 6). The amount of free cytochrome c oxidase was decreased upon depletion of PE (Fig. 4b, lanes 2, 3, and 6), indicating that the lack of PE stabilizes the interaction of both complexes. For comparison, lack of CL in crd1Δ mitochondria has an opposing effect on the supercomplexes as the association of the cytochrome bc1 complex with cytochrome c oxidase is destabilized (Fig. 4a, lane 5; Fig. 4b, lane 8).38,39,47,48,82,88,89

Neither succinate dehydrogenase (complex II) nor the F1F0-ATP synthase (complex V) altered their blue native mobility upon depletion of PE (Fig. 4c), indicating that these complexes were not present in the large oligomeric assembly of the respiratory chain.

In conclusion, PE and CL are both important for mitochondrial function. Like CL, PE is required for maintaining the membrane potential, which is crucial for the import of preproteins into and across the inner membrane. The activity of the respiratory chain, in particular of cytochrome c oxidase, is decreased when PE (this study) or CL48,49 is depleted. However, PE and CL showed different effects on the molecular level when the stability of mitochondrial protein complexes was analyzed. Lack of CL results in dissociation of AAC oligomers and destabilization of respiratory chain supercomplexes.35,36,39,48,49 In contrast, upon depletion of PE, the AAC oligomers remained stable and even higher forms of the respiratory chain supercomplexes were observed. CL and PE were shown to bind to the cytochrome bc1 complex.

Fig. 4. Depletion of PE stabilizes respiratory chain supercomplexes. (a–c) Wild-type, psd1Δ, psd1Δ psd2Δ, and crd1Δ mitochondria were lysed with 1% (w/v) digitonin in digitonin buffer and subjected to blue native electrophoresis, followed by Western blotting using the indicated antisera. III, cytochrome bc1 complex; IV, cytochrome c oxidase; V2, ATP synthase dimer; V, ATP synthase monomer; Atp4, ATP synthase subunit 4 (subunit b); Cox4, cytochrome c oxidase subunit 4; Cox6, cytochrome c oxidase subunit 6; Rip1, Rieske iron–sulfur protein; SDH, succinate dehydrogenase (complex II).
and cytochrome c oxidase, likely including the interface of both complexes. \cite{12,43,45,64,90,91} CL and PE are both non-bilayer-forming phospholipids. Their opposite effects on protein complex stability may provide an explanation why CL does not fully compensate for the loss of PE and vice versa. CL has a negatively charged head group, whereas PE is a zwitterionic phospholipid of neutral charge, suggesting that the differently charged head groups may contribute to the differential effects on protein complex stability. Wenz et al. indeed showed that the negative charge of CL is important for maintaining the structural integrity of respiratory supercomplexes. \cite{91} Wittig and Schägger\cite{92} and Bultema et al.\cite{93} proposed that the cytochrome bc\(_1\) complex and the cytochrome c oxidase can be organized into higher oligomeric structures that are larger than the known supercomplexes and called them respiratory strings or megacomplexes. Our results suggest that such structures are stabilized when PE is depleted and thus megacomplexes of the respiratory chain can be detected on blue native gels.

Acknowledgements

We thank Drs. Natalia Gebert, Martin van der Laan, and Nora Vögtle for materials and discussion and Nicole Zufall for expert technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 746, Excellence Initiative of the German Federal and State Governments (EXC 294 BIOSS; GSC-4 Spemann Graduate School), Bundesministerium für Bildung und Forschung, the Austrian Science Fund FWF (project 21429), and DK Molecular Enzymology (W901-B05 to G.D.).

Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2012.09.001

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**Additional citations**


