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Phosphatidylcholine Affects Inner Membrane Protein Translocases of Mitochondria*

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Two protein translocases transport precursor proteins into or across the inner mitochondrial membrane. The presequence translocase (TIM23 complex) sorts precursor proteins with a cleavable presequence either into the matrix or into the inner membrane. The carrier translocase (TIM22 complex) inserts multispanning proteins into the inner membrane. Both protein import pathways depend on the presence of a membrane potential, which is generated by the activity of the respiratory chain. The non-bilayer-forming phospholipids cardiolipin and phosphatidylethanolamine are required for the activity of the respiratory chain and therefore to maintain the membrane potential for protein import. Depletion of cardiolipin further affects the stability of the TIM23 complex. The role of bilayer-forming phospholipids like phosphatidylcholine (PC) in protein transport into the inner membrane and the matrix is unknown. Here, we report that import of presequence-containing precursors and carrier proteins is impaired in PC-deficient mitochondria. Surprisingly, depletion of PC does not affect stability and activity of respiratory supercomplexes, and the membrane potential is maintained. Instead, the dynamic TIM23 complex is destabilized when the PC levels are reduced, whereas the TIM22 complex remains intact. Our analysis further revealed that initial precursor binding to the TIM23 complex is impaired in PC-deficient mitochondria. We conclude that reduced PC levels differentially affect the TIM22 and TIM23 complexes in mitochondrial protein transport.

Mitochondria fulfill essential functions for the survival of the cell like energy conversion to produce ATP, synthesis of amino acids, lipids, and heme, as well as the generation of iron-sulfur clusters. They contain about 1000 proteins in yeast and 1500 proteins in humans (1, 2). More than 99% of the mitochondrial proteins are synthesized as precursors on cytosolic ribosomes. Mitochondria contain a sophisticated system of protein translocases to import precursor proteins (3–9). The translocase of the outer membrane (TOM[3] complex) forms the general entry gate for most precursor proteins. After passage of the TOM channel, distinct protein translocases sort the preproteins into the different subcompartments: the outer and inner membrane as well as the two aqueous compartments, the matrix and intermembrane space.

The majority of mitochondrial proteins are sorted into the inner membrane and the matrix. Two inner membrane-bound protein complexes mediate protein import. The presequence translocase (also termed TIM23 complex) transports precursor proteins with a cleavable presequence into the inner membrane and the matrix, whereas the carrier translocase (also termed TIM22 complex) inserts proteins with multiple transmembrane segments into the inner membrane (3–9). The membrane potential across the inner membrane provides the driving force for both protein import pathways and is generated by the activity of the respiratory chain. Presequence-containing preproteins are directly transferred from the TOM complex to the TIM23 complex (10, 11). The TIM23 complex consists of five subunits: Tim23 forms the translocation channel, which is in close association with Tim17 (12, 13). The intermembrane space-exposed domains of Tim23, Tim50, and Tim21 facilitate preprotein transfer from the TOM complex to the Tim23 channel (14–17). The fifth TIM23 subunit Mgr2 (mitochondrial genome required) controls sorting of preproteins into the inner membrane and stabilizes the association of Tim21 with the translocase (18, 19). The TIM23 complex laterally releases precursor proteins into the inner membrane (14, 20). For transport into the mitochondrial matrix, the TIM23 complex dynamically associates with the presequence translocase-associated motor (PAM). The ATP consuming activity of the mitochondrial Hsp70 within the PAM module completes preprotein transport into the matrix (3–9). Finally, the presequence is removed by the mitochondrial processing peptidase. Precursors of carrier proteins lack such a cleavable presequence. Small TIM chaperones guide these hydrophobic preproteins from the TOM complex to the carrier translocase. The

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[3] The abbreviations used are: TOM, translocase of the outer membrane; TIM23, presequence translocase of the inner membrane; TIM22, carrier translocase of the inner membrane; SAM, sorting and assembly machinery; CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PAM, presequence translocase-associated motor; $DISC_3$, 3,3-dipropylthiadicarbocyanine iodide; AAC, ADP-ATP carrier; DHFR, dihydrofolate reductase.

TIM22 complex consists of four membrane-bound subunits. Tim54 mediates the docking of the preprotein-loaded small TIM chaperones to the carrier translocase (21–23). Tim22 forms a twin-pore to insert preproteins into the inner membrane (24–26). Finally, Tim18 and Sdh3 are required to warrant assembly and stability of the TIM22 complex (27–29).

Mitochondrial membranes contain five major phospholipids: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol, phosphatidylserine, and cardiolipin (CL). The majority of these phospholipids are synthesized in the endoplasmic reticulum. Mitochondria are also able to generate a limited number of lipids. CL and PE are synthesized in the inner mitochondrial membrane (30–34). The CL biosynthesis pathway consists of multiple steps (30–34), whereas PE is produced by decarboxylation of phosphatidylserine by the phosphatidylserine decarboxylase 1 (Psd1) (35–37). In yeast, Psd1 is the major source of cellular PE under standard growth conditions (38). However, further sources for cellular PE exist. Free ethanolamine is converted to CDP-ethanolamine that is subsequently transferred onto diacylglycerol to form PE (Kennedy pathway). Psd2 of endosomes and the activity of the acyltransferases Tgl3 and Ale2 produce additional amounts of PE (38–42). Isolated CL and PE do not form membrane bilayer structures and are regarded as non-bilayer-forming lipids (30, 43). Both phospholipids are crucial for mitochondrial function and morphology (44–46). Double deletion of Psd1 with the cardiolipin synthase Crd1 is lethal confirming the closely related functions of CL and PE (47).

Studies done over the last few years revealed specific roles of CL and PE in protein transport into mitochondrial subcompartments (30, 31, 48). In mutants defective in CL or PE synthesis, the biogenesis of outer membrane β-barrel proteins is affected (49, 50). Whereas CL is also required for the import of proteins with multiple α-helical membrane spans into the outer membrane, this import pathway remains largely unaffected in PE-deficient mitochondria (50, 51). Furthermore, both phospholipids promote protein transport into the inner membrane and matrix (52–57). First, binding of preproteins to the TOM complex is disturbed in PE- and CL-deficient mitochondria (49, 50). Second, the activity of the respiratory chain complexes, in particular of the cytochrome *c* oxidase (complex IV), is decreased in mitochondria with reduced PE or CL content (57–59). Consequently, the membrane potential is decreased, which leads to reduced protein translocation via TIM23 or TIM22 translocases (52, 55, 57). CL and PE exhibit distinct roles in the stability of protein complexes. Whereas deletion of CL affects the stability of respiratory chain supercomplexes as well as of the TOM and TIM23 translocases, these protein complexes remain largely intact in PE-deficient mitochondria (49, 50, 53–58, 60–62). CL associates with respiratory chain complexes (63, 64) and stabilizes the interaction of the cytochrome *c* reductase (complex III) and complex IV via its negatively charged headgroup (65).

The role of bilayer-forming phospholipids in mitochondrial functions is poorly understood. Phosphatidylcholine (PC) is the most abundant phospholipid of the mitochondrial membranes (66, 67). Two pathways in the endoplasmic reticulum produce cellular PC. First, within the Kennedy pathway free choline is activated via phosphorylation and subsequent binding to CDP. CDP-choline is then linked to diacylglycerol to form PC (31, 68). Second, PE can be methylated in three steps to produce PC. Pem1/Cho2 promotes the first methylation step, whereas Pem2/Opi3 is capable of performing all three methylation steps but the last two with higher efficiency (69–74). PC is essential for the survival of the cell (72). Recent studies revealed that yeast cells with decreased PC levels show a reduced growth under non-fermentative conditions and that the biogenesis of outer membrane β-barrel and some α-helical proteins is impaired in mitochondria isolated from these cells (75). The role of PC in protein transport into the inner mitochondrial subcompartments is unknown.

We studied protein transport into the inner membrane and matrix in mitochondria isolated from mutants defective in PC biosynthesis. We found that the import of both precursors with a cleavable presequence and carrier proteins is reduced in the mutant mitochondria. Strikingly, depletion of PC does not affect the activity of the respiratory chain, and the membrane potential across the inner membrane is maintained in these mutants. Decreased PC levels differentially affect the stability of TIM23 and TIM22 translocases. Whereas the TIM23 translocase is destabilized, the TIM22 complex remains intact. Although the TOM complex is functional, the arrest of a preprotein in the TOM-TIM23 supercomplex is impaired. We conclude that PC affects initial binding of precursor proteins to the TIM23 translocase. Altogether, depletion of PC specifically affects the function of inner membrane protein translocases of mitochondria.

Results

Depletion of PC Impairs Protein Transport into the Inner Membrane and the Matrix—To study the role of PC in protein transport into and across the inner mitochondrial membrane, we chose *pem1*Δ and *pem2*Δ mutant strains, which are defective in the methylation pathway of PE to produce PC (69–74). Yeast cells were grown in minimal medium to block the synthesis of PC from free choline via the Kennedy pathway. We used a non-fermentable carbon source to promote mitochondrial function. We determined the phospholipid profiles in total cell extract and in isolated mitochondria. The relative amounts of PC were strongly reduced in the cell extract and mitochondria of both mutants but more severely in *pem2*Δ mitochondria (Fig. 1*A*). As reported, the content of PE was drastically increased (Fig. 1*A*) (47, 69–72, 75). In *pem2*Δ mitochondria, the monomethylated form of PE accumulated (47, 69–72) but was not separated here from PE. For comparison, we determined the phospholipid profile of *psd1*Δ mutant cells, which were grown under the same conditions like *pem1*Δ and *pem2*Δ cells. Mitochondrial PE was strongly reduced in the *psd1*Δ mutant, whereas the relative levels of total cellular PE were mildly decreased (Fig. 1*A*). In contrast, the PC content was strongly increased in total cell extracts and mitochondria of the *psd1*Δ mutant compared with wild-type cells (Fig. 1*A*). Under these growth conditions, PC was synthesized by methylation of PE that is predominantly produced by Psd2 (38). Thus, it is crucial for the yeast cells to maintain certain amounts of PC/PE, although the ratio can vary drastically. In the PC- and PE-defi-

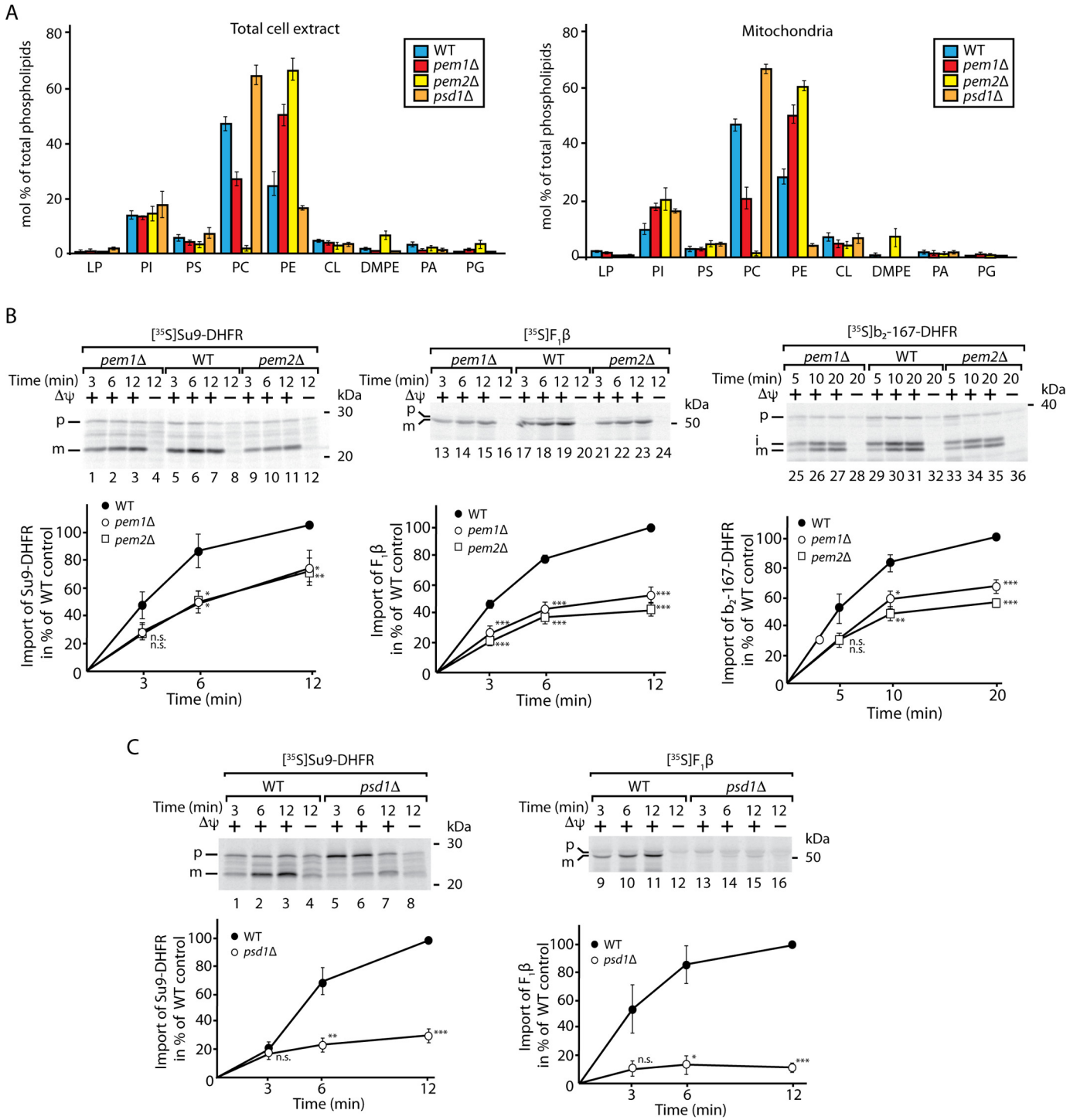

FIGURE 1. **Import of presequence-containing precursor proteins into the mitochondrial inner membrane and matrix is affected in PC-deficient mitochondria.** *A,* relative amounts of phospholipids from total cell extracts (*left panel*) and isolated mitochondria (*right panel*) from *pem1*Δ, *pem2*Δ, and *psd1*Δ cells were determined. Depicted are mean values of three independent experiments with their corresponding S.E. *LP,* lyso-phospholipids; *DMPE,* dimethyl-phosphatidylethanolamine; *PA,* phosphatidic acid; *PG,* phosphatidylglycerol; *PI,* phosphatidylinositol, *PS*; phosphatidylserine. *B,* ^{35}S-labeled precursors of Su9-DHFR, $F_1\beta$, and cytochrome b_2-167-DHFR were imported for the indicated time periods into wild-type (*WT*), *pem1*Δ, and *pem2*Δ mitochondria. Non-imported precursor proteins were proteolytically removed by proteinase K. *Upper panel,* the import reaction was analyzed by SDS-PAGE and autoradiography. *p,* precursor form; *i,* import intermediate; *m,* mature protein. *Lower panel,* quantifications of the import reactions of the *upper panel* are shown. Depicted are the mean values and their corresponding S.E. of seven (Su9-DHFR), six ($F_1\beta$), and four (b_2-167-DHFR) independent import experiments. Statistically significant differences based on an unpaired *t* test of the individual import time points in mutant mitochondria related to wild-type control are depicted (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *n.s.*, not significant). *C,* ^{35}S-labeled precursors of Su9-DHFR and $F_1\beta$ were imported for the indicated time periods into wild-type (*WT*) and *psd1*Δ mitochondria. Non-imported precursor proteins were proteolytically removed by proteinase K. *Upper panel,* the import reaction was analyzed by SDS-PAGE and autoradiography. *p,* precursor form; *i,* import intermediate; *m,* mature protein. *Lower panel,* quantifications of the import reactions of the upper *panel* are shown. Depicted are the mean values and their corresponding S.E. of four (Su9-DHFR) and three ($F_1\beta$) independent import experiments. Statistically significant differences based on an unpaired *t* test of the individual import time points in mutant mitochondria related to wild-type control are depicted (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *n.s.*, not significant).

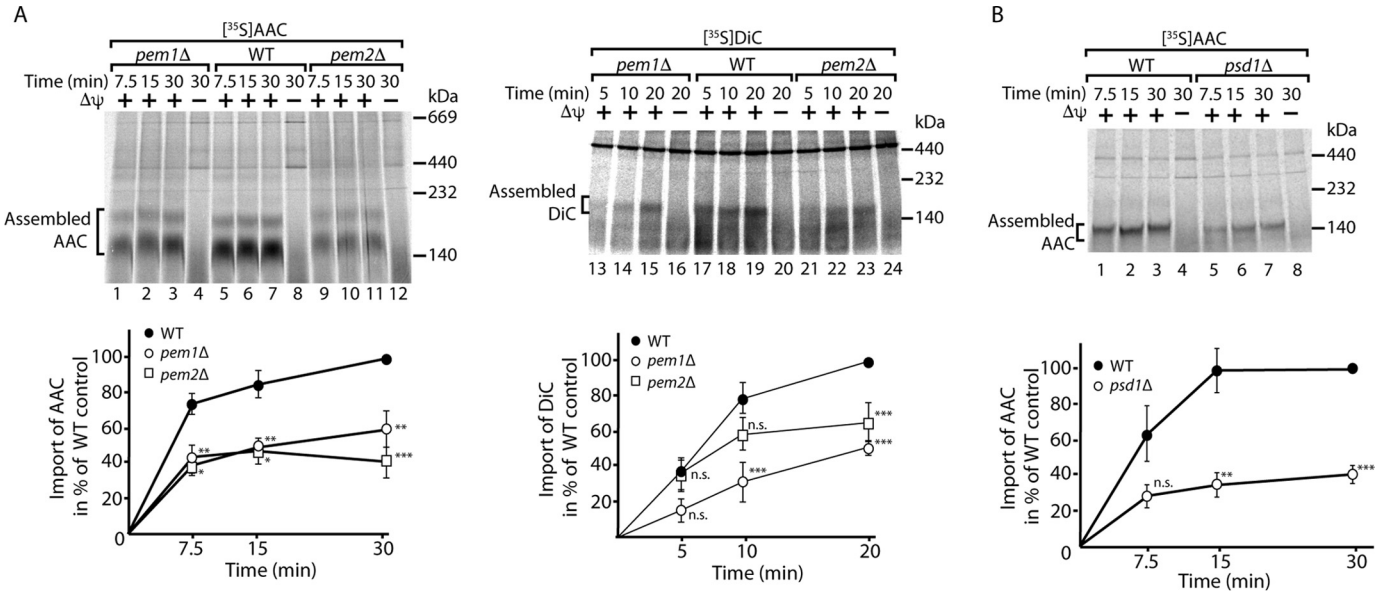

FIGURE 2. **Import of carrier proteins into the mitochondrial inner membrane and matrix is affected in PC-deficient mitochondria.** *A,* ^{35}S-labeled precursors of AAC and dicarboxylate carrier (*DiC*) were imported for the indicated time periods into wild-type (*WT*), *pem1*Δ, and *pem2*Δ mitochondria. *Upper panel*, the import reaction was analyzed by blue native electrophoresis and autoradiography. *Lower panel*, quantifications of the import reactions of the *upper panel* are shown. Depicted are the mean values and their corresponding S.E. of five independent import experiments. Statistically significant differences based on an unpaired *t* test of the individual import time points in mutant mitochondria related to wild-type control are depicted (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *n.s.,* not significant). *B,* ^{35}S-labeled precursor of AAC was imported for the indicated time periods into wild-type (*WT*) and *psd1*Δ mitochondria. *Upper panel*, the import reaction was analyzed by blue native electrophoresis and autoradiography. *Lower panel*, quantifications of the import reactions of the *upper panel* are shown. Depicted are the mean values and their corresponding S.E. value of five independent import experiments. Statistically significant differences based on an unpaired *t* test of the individual import time points in mutant mitochondria related to wild-type control are depicted (**, $p < 0.01$; ***, $p < 0.001$; *n.s.,* not significant).

cient mutants, the mitochondrial phosphatidylinositol levels are moderately increased, whereas the content of other phospholipids remains largely comparable with wild type (Fig. 1*A*).

We isolated mitochondria from *pem1*Δ and *pem2*Δ to analyze whether reduced PC levels affect import of presequence-containing proteins into mitochondria. We chose three model precursor proteins for our studies. Precursors of the model preprotein Su9-DHFR and of the $F_1\beta$-subunit of the F_1F_0-ATP synthase are transported into the matrix, whereas the precursor of cytochrome b_2-DHFR is sorted into the inner membrane. Radiolabeled precursor proteins were synthesized and incubated with isolated mitochondria. Non-imported precursor proteins were removed by addition of proteinase K. Successful translocation into mitochondria can be monitored by the detection of the mature band, which is formed after proteolytic removal of the presequence by the mitochondrial processing peptidase. The precursor of cytochrome b_2-DHFR is processed in a second step by Imp1 (76), which results in the presence of an intermediate band. The import of all three precursor proteins was moderately reduced in *pem1*Δ and *pem2*Δ mitochondria (Fig. 1*B*). For comparison, imports of Su9-DHFR and F1β-subunit were strongly impaired in *psd1*Δ (Fig. 1*C*), pointing to distinct modes of how PC and PE affect TIM23-dependent protein translocation.

Carrier proteins lack a cleavable presequence. To determine the import of the ADP-ATP carrier (AAC) and of the dicarboxylate carrier, we monitored their assembly into the inner membrane. After the import reaction, mitochondria were solubilized with the mild detergent digitonin, and protein complexes were separated by blue native electrophoresis. The import of both carrier proteins was impaired in *pem1*Δ and *pem2*Δ mitochondria (Fig. 2*A*). The import of the AAC precursor was strongly affected in *psd1*Δ mitochondria (Fig. 2*B*). Altogether, we conclude that reduced PC levels affect protein import via the presequence and carrier pathway.

PC Is Not Required for Stability and Activity of the Respiratory Chain—Two scenarios are conceivable to explain the defective protein import into and across the inner mitochondrial membrane in the PC-deficient mitochondria. First, the membrane potential could be decreased, which would cause a delayed protein transport. Second, the functions of the TIM23 and TIM22 translocases could be disturbed. To experimentally address the first possibility, we determined the membrane potential of isolated mitochondria. In this assay, a fluorescent dye is taken up by isolated mitochondria in a membrane potential-dependent manner, which results in a quenching of the fluorescence signal (52). Fluorescence signals close to wild-type mitochondria indicate that the membrane potential is intact. Reduced membrane potential results in less quenching of the fluorescence compared with the wild-type control. Addition of the ionophore valinomycin dissipates the membrane potential, which causes a release of the dye from wild-type and mutant mitochondria and restoration of the fluorescent signal in all samples (Fig. 3). Unexpectedly, following this experimental strategy we observed that the membrane potential remained largely unaffected in *pem1*Δ and *pem2*Δ compared with wild-type mitochondria (Fig. 2). Thus, a dissipated membrane potential does not impair protein import into or across the inner membrane of PC-deficient mito-

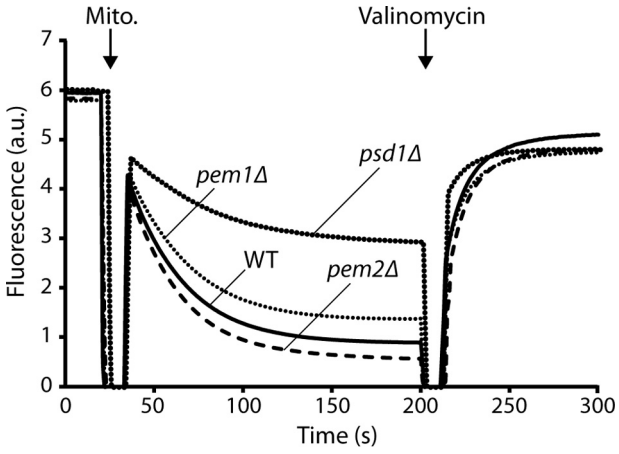

FIGURE 3. **Membrane potential across the inner mitochondrial membrane is not affected in PC-deficient mitochondria.** The membrane potential of wild-type (*WT*), *pem1*Δ, *pem2*Δ, and *psd1*Δ mitochondria (*Mito.*) was determined by the membrane potential-dependent uptake of the fluorescent dye $DISC_3$ into mitochondria as described under "Experimental Procedures." Where indicated, valinomycin was added to release $DISC_3$ from mitochondria (*Mito.*). Mean values of three independent measurements are depicted.

chondria. In contrast, the membrane potential was compromised in mitochondria isolated from *psd1*Δ cells (Fig. 3) as reported previously (57). The diminished membrane potential in *psd1*Δ mitochondria leads to strongly impaired protein import via the TIM23 and TIM22 complexes (Figs. 1*C* and 2*B*). We conclude that the membrane potential was differentially affected by depletion of PC or PE.

The non-bilayer forming phospholipids PE and CL are required for full activity of the respiratory chain, which generates the membrane potential across the inner membrane (52, 55, 57–59). Thus, we wondered whether the respiratory chain complexes were present and functional in PC-deficient mitochondria. The steady state levels of various subunits of the respiratory chain were largely unchanged in mutant compared with wild-type mitochondria (Fig. 4*A*). We only observed a mild reduction of a few components of the cytochrome *c* oxidase (complex IV) (Fig. 4*A*). Cytochrome *c* reductase (complex III) and complex IV of the respiratory chain form supercomplexes, which can be analyzed by blue native electrophoresis. In yeast, one or two copies of complex IV associate with a dimer of complex III (77, 78). We found that these supercomplexes were present in *pem1*Δ and *pem2*Δ as in wild-type mitochondria (Fig. 4*B*, *lanes 1–15*). Furthermore, the reduced PC content did not affect the stability of the succinate dehydrogenase (complex II) and of the F_1F_0-ATP synthase (Fig. 4*B*, *lanes 16–21*). We conclude that the respiratory chain complexes are not destabilized in *pem1*Δ and *pem2*Δ mitochondria. In *psd1*Δ mitochondria, the respiratory chain supercomplexes are stable, but the activity of particular complex IV is decreased (57). To directly visualize the activity of respiratory chain supercomplexes, we performed in-gel activity stain of complex IV (79). Strikingly, the amount of active complex IV was not reduced in *pem1*Δ and *pem2*Δ mitochondria (Fig. 4*C*). Similarly, the activity of the monomeric and dimeric F_1F_0-ATP synthase (complex V) was not affected in the mutant mitochondria (Fig. 4*D*). We conclude that depletion of PC does not affect the stability and function of the respiratory chain supercomplexes. Supporting our findings, a recent study reported that respiratory chain supercomplexes are functional and stable in PC-deficient mitochondria (80). We conclude that the respiratory chain is able to establish a membrane potential in PC-deficient mitochondria, which is comparable with wild-type mitochondria.

Depletion of PC Differentially Affects the Stability of Inner Membrane Protein Translocases—We excluded the possibility that a decreased membrane potential caused the defective protein import across and into the inner membrane of PC-depleted mitochondria. Therefore, we asked whether the stability of the protein translocases was affected in PC-deficient mutant mitochondria. The steady state levels of various subunits of the TIM23 and TIM22 translocases as well as of control proteins were unchanged in *pem1*Δ and *pem2*Δ in comparison with wild-type mitochondria (Fig. 5*A*). Notably, the steady state levels of Tim10 were largely unaffected in these mutant mitochondria as well (Fig. 5*A*) (75). Small TIM chaperones like Tim10 are essential for transport of carrier precursors through the intermembrane space to the TIM22 translocase (22, 23). Next, we analyzed the stability of the protein translocases by blue native electrophoresis and Western blotting. The TIM23 translocase is a highly dynamic protein complex, which forms two complexes on a blue native gel. The TIM23 core complex consists of Tim23, Tim17, and Tim50, whereas the TIM23 sorting form additionally contains Tim21 (14, 53). Strikingly, in *pem1*Δ and *pem2*Δ mitochondria, both TIM23 forms were reduced when detected with antibodies raised against Tim17 or Tim23 (Fig. 5*B*). Thus, depletion of PC affects the integrity of the TIM23 translocase. In contrast, the formation of the TIM22 complex remained largely unaltered in *pem1*Δ and *pem2*Δ mitochondria as shown by detection with antibodies specific for Tim22 and Tim54 (Fig. 5*C*). As control, the mitochondrial intermembrane space import and assembly machinery (MIA), the TOM complex, and the Hsp60 ring complexes were normally formed in the mutant mitochondria (Fig. 5*D*). We conclude that depletion of PC differentially affects the stability of the inner membrane protein translocases. Whereas the dynamic TIM23 complex is destabilized, the carrier translocase is formed normally.

Depletion of PC Affects Precursor Transfer to the TIM23 Complex—Precursor proteins are first transported across the outer membrane via the TOM complex and then directly transferred to the TIM23 translocase. We wondered whether the initial binding of the precursor to the TIM23 complex is affected in PC-deficient mitochondria. To this end, we arrested a variant of the precursor of cytochrome b_2 (b_2(167)Δ-DHFR) that lacks the inner membrane-sorting signal at an early import stage. In the presence of a membrane potential and methotrexate, the radiolabeled cytochrome b_2 portion passes the TOM complex and engages the TIM23 complex. Addition of methotrexate induces a stable folding of the DHFR moiety, which blocks its passage through the TOM channel. Consequently, the cytochrome b_2(167)Δ-DHFR construct gets arrested in the TOM-TIM23 supercomplex, which can be analyzed by blue native electrophoresis (10, 11). Strikingly, the accumulation of this precursor in the TOM-TIM23 supercomplex was

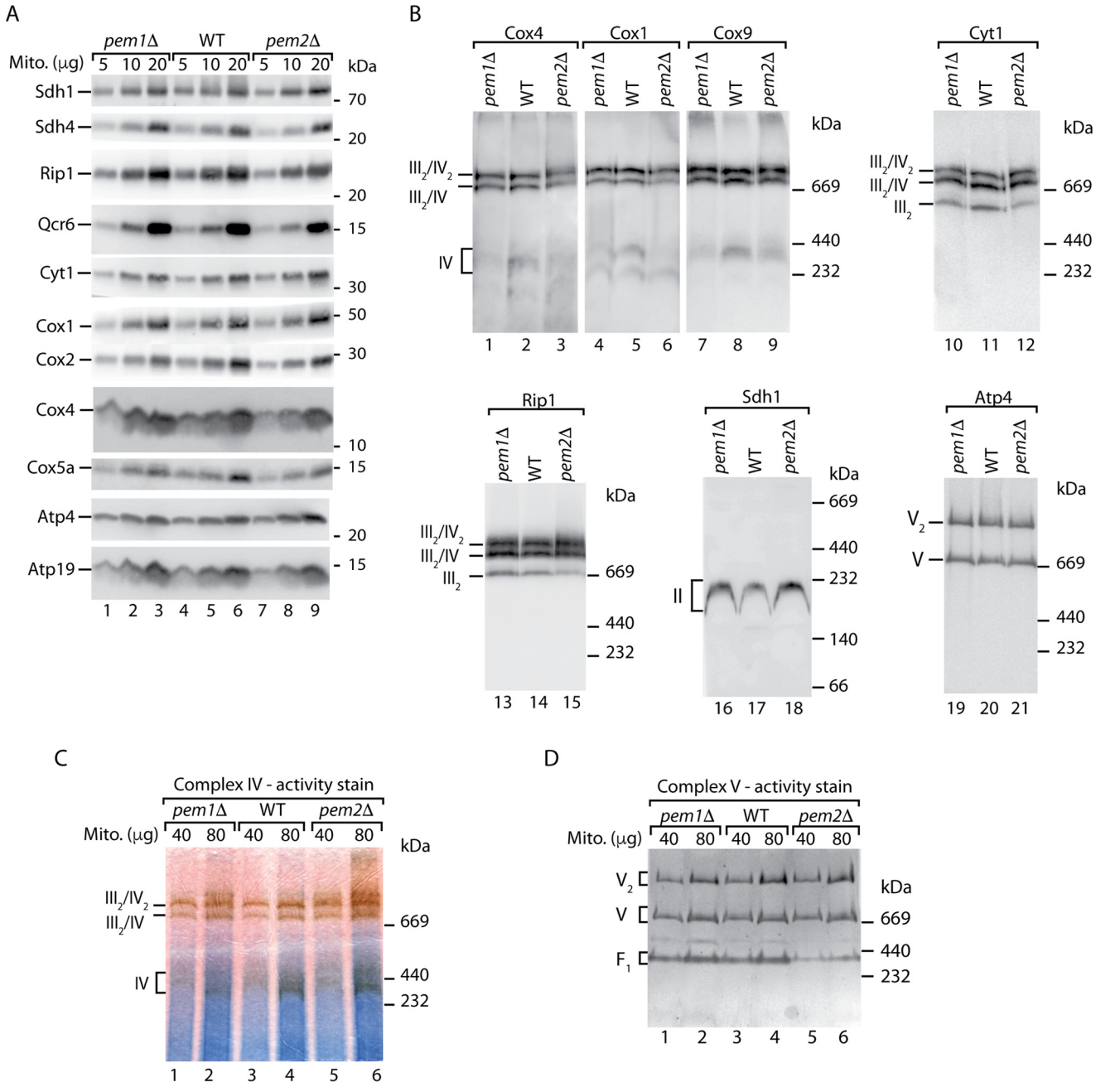

FIGURE 4. **Depletion of PC does not perturb stability and activity of the respiratory chain supercomplexes.** *A,* indicated amounts of mitochondrial proteins from wild-type (*WT*), *pem1*Δ, and *pem2*Δ cells were separated by SDS-PAGE and detected by immunodetection with the indicated antisera. *Sdh*, succinate dehydrogenase; *Qcr6*, subunit 6 of the ubiquinol-cytochrome *c* oxidoreductase (complex III); *Rip1*, Rieske iron-sulfur protein 1 (complex III); *Cox*, subunit of the cytochrome *c* oxidase (complex IV); *Atp*, subunit of the F_1F_0-ATP synthase (complex V). *Mito.*, mitochondria. *B,* WT, *pem1*Δ, and *pem2*Δ mitochondria were lysed under native conditions and subjected to blue native electrophoresis. Protein complexes were analyzed by immunodetection with the indicated antisera. *C,* WT, *pem1*Δ, and *pem2*Δ mitochondria were lysed under native conditions and subjected to blue native electrophoresis. The activity of complex IV (cytochrome *c* oxidase) was detected by in-gel activity stain. *D,* WT, *pem1*Δ, and *pem2*Δ mitochondria were lysed under native conditions and subjected to blue native electrophoresis. The activity of complex V (F_1F_0-ATP synthase) was detected by in-gel activity stain.

decreased in both mutant mitochondria but particularly in *pem2*Δ mitochondria (Fig. 6*A*). One possibility is that the reduced PC levels affect the function of the TOM complex. However, the accumulation of TIM23-dependent precursors like Om45-DHFR (81, 82) and Oxa1 at the TOM complex was not decreased (Fig. 6*B*) (75). Furthermore, the TOM complex remains intact in PC-deficient mitochondria (Fig. 5*D*) (75). We conclude that depletion of PC affects initial recognition of the precursor by the TIM23 complex. To investigate whether the import of mitochondrial carrier proteins was affected at the TOM stage, we imported AAC fused to DHFR (AAC-DHFR) into isolated mutant mitochondria. Upon import, the majority of the precursor binds to the TOM complex but is not further transported into the inner membrane (83, 84). Imported AAC-DHFR precursor efficiently accumulated at the TOM complex in *pem1*Δ and *pem2*Δ mitochondria (Fig. 6*C*), indicating that the binding of the carrier protein to the TOM complex was not compromised. We conclude that a reduced PC content does

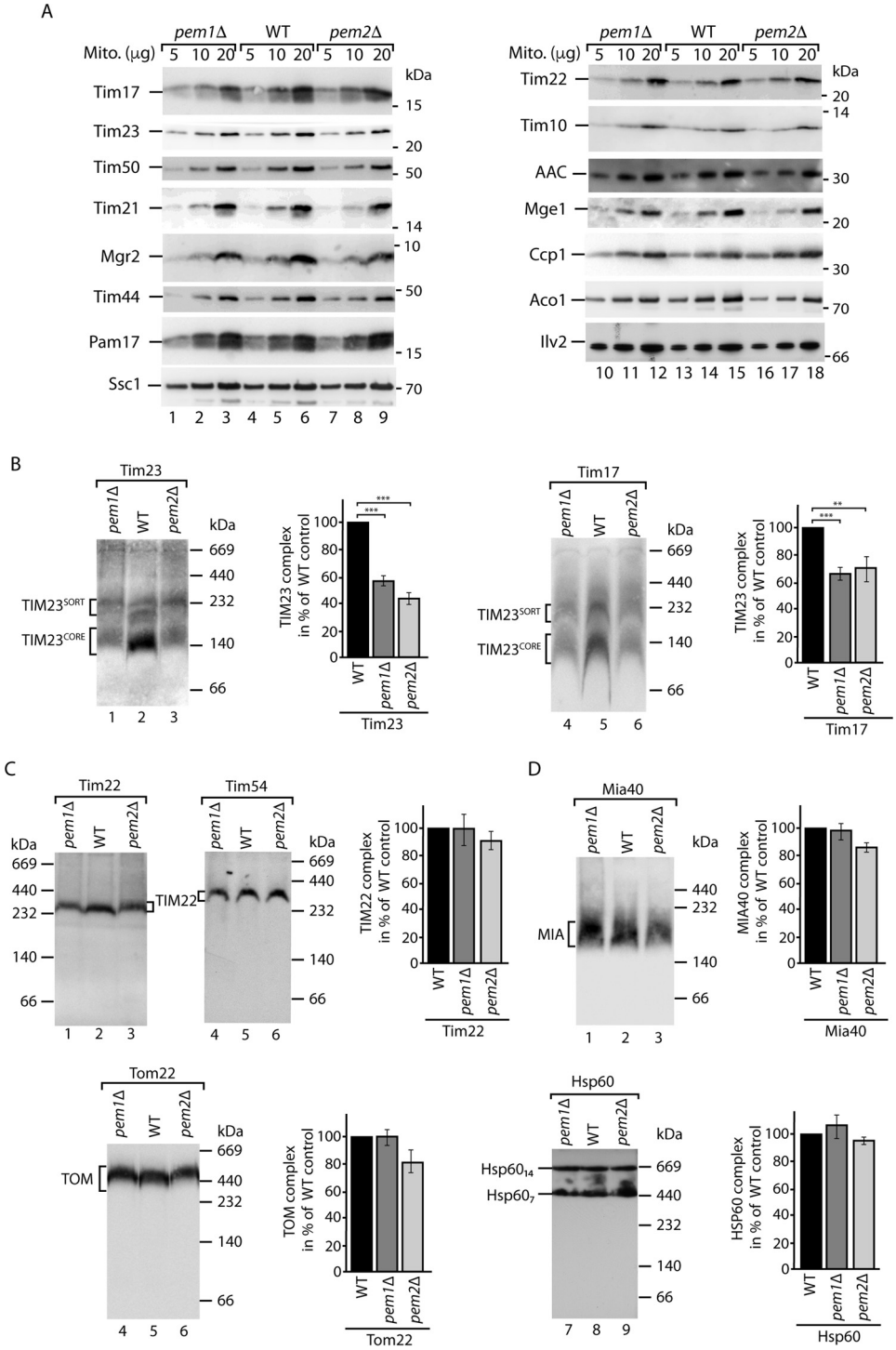

FIGURE 5. **Depletion of PC differentially affects the stability of inner membrane protein translocases.** *A,* indicated amounts of mitochondrial proteins from wild-type (*WT*), *pem1*Δ, and *pem2*Δ cells were separated by SDS-PAGE and detected by immunodetection with the indicated antisera. *Mgr2*, mitochondrial genome required; *AAC*, ADP-ATP carrier; *Mge1*, mitochondrial GrpE; *Ccp1*, cytochrome *c* peroxidase 1; *Aco1*, aconitase; *Ilv2*, isoleucine plus valine requiring. *B–D,* WT, *pem1*Δ, and *pem2*Δ mitochondria (*Mito.*) were lysed under native conditions and subjected to blue native electrophoresis. Protein complexes were analyzed by immunodetection with the indicated antisera. *TIM23*, presequence translocase; *TIM22*, carrier translocase; *MIA*, mitochondrial intermembrane space import and assembly; *TOM*, translocase of the outer membrane; $Hsp60_7$, Hsp60 heptamer; $Hsp60_{14}$, Hsp60 tetradecamer. Quantifications of the protein complexes were shown. Depicted are the mean values with their corresponding S.E. of four ($Hsp60_{14}$), seven (Tim17 (TIM23core)), Tim22, Mia40, eight (Tim23 (Tim23core)), and nine (Tom22) independent experiments. Statistically significant differences based on an unpaired *t* test for the TIM23 complex in mutant mitochondria related to wild-type control are depicted (**, $p < 0.01$; ***, $p < 0.001$).

not affect initial binding of the carrier precursor to the TOM complex but impairs the import of the precursor into the inner membrane by the TIM22 complex. Altogether, transport of precursor proteins into the inner membrane and the matrix is affected at the stage of the TIM translocases in PC-deficient mitochondria.

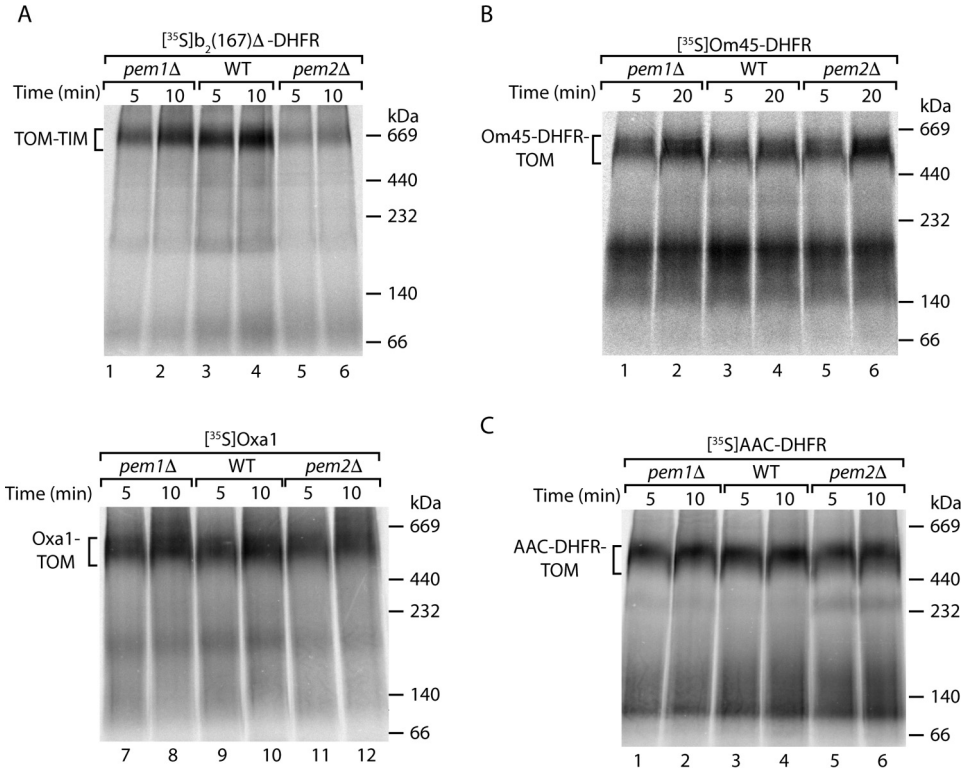

FIGURE 6. **Arrest of a precursor protein in the TOM-TIM23 supercomplex is defective in PC-deficient mitochondria.** *A,* ^{35}S-labeled precursors of cytochrome b_2(167)Δ-DHFR were imported for the indicated time periods into wild-type (*WT*), *pem1*Δ, and *pem2*Δ mitochondria in the presence of methotrexate. The import reaction was analyzed by blue native electrophoresis and autoradiography. *B,* ^{35}S-labeled precursors of Om45-DHFR and Oxa1 were imported for the indicated time periods into wild-type (*WT*), *pem1*Δ, and *pem2*Δ mitochondria. The import of Om45-DHFR was performed in the presence of methotrexate. The arrest of the Oxa1 precursor at the TOM complex was studied after dissipation of the membrane potential. The samples were analyzed by blue native electrophoresis and autoradiography. *C,* ^{35}S-labeled precursors of AAC-DHFR were imported for the indicated time periods into wild-type (*WT*), *pem1*Δ, and *pem2*Δ mitochondria in the presence of methotrexate. The import reaction was analyzed by blue native electrophoresis and autoradiography.

Discussion

We report that depletion of PC affects protein import into the mitochondrial inner membrane and matrix. Surprisingly, the stability and activity of respiratory chain supercomplexes are not reduced in mitochondria isolated from *pem1*Δ and *pem2*Δ cells. Consequently, the membrane potential generated by the respiratory chain is sufficient to drive protein transport in the mutant mitochondria. We demonstrate that the TIM23 complex is destabilized and that preprotein arrest in a TOM-TIM23 supercomplex is impaired in PC-deficient mutant mitochondria. In contrast, accumulation of TIM23- and TIM22-dependent precursor proteins at the TOM complex is not altered. We conclude that PC affects protein transport into and across the inner membrane at the stage of the TIM translocases.

Our studies on mitochondrial protein biogenesis revealed unexpected specific effects of reduced PC levels on protein machineries. Depletion of PC selectively affects the stability of highly dynamic protein translocases like the TIM23 complex of the inner membrane and the sorting and assembly machinery (SAM complex) of the outer membrane (75). The SAM complex mediates folding and insertion of β-barrel proteins in the mitochondrial outer membrane (3–5, 7, 8). It interacts with different partner proteins to promote protein biogenesis. First, the SAM complex interacts with the TOM complex to facilitate transfer of β-barrel precursors (76, 85). Second, it associates with the mitochondrial division and morphology protein Mdm10 to promote the assembly of Tom22 into the TOM complex (86–88). Depletion of PC affects both the biogenesis of β-barrel proteins and the assembly of Tom22 (75). The initial binding of the β-barrel precursor to the TOM-SAM supercomplex is decreased in PC-depleted mitochondria (75). The TIM23 complex mediates protein transport into the matrix and lateral release into the inner membrane. TIM23 subunits dynamically interact with the PAM module for transport into the matrix (3–9). Furthermore, the TIM23 complex interacts with the TOM complex during preprotein transfer. Precursor proteins can be arrested experimentally in a TOM-TIM23 supercomplex (10, 11, 14). In PC-deficient mitochondria, the stability of the TIM23 complex is decreased, and both TIM23-dependent import pathways into the matrix and inner membrane are affected. Furthermore, binding of a precursor protein to the TOM-TIM23 supercomplex is impaired upon depletion of PC. Because precursor accumulation at the TOM complex remains unaffected, the initial binding of preproteins by the TIM23 complex is impaired in mutants defective in PC biosynthesis. In contrast to the TIM23 complex, the TOM translocase and the respiratory chain supercomplexes are not destabilized in PC-deficient mitochondria (75). Moreover, the integration of the SAM-independent model precursors Tom20 and Om45 are not impaired in mitochondria with reduced PC levels (75). We conclude that depletion of PC selectively affects distinct protein transport pathways but does not generally compromise protein import into mitochondria. This is an unexpected find-

ing because PC is the most abundant phospholipid in mitochondrial membranes.

Previous studies revealed specific roles of the non-bilayer forming phospholipids PE and CL in protein import into and across both mitochondrial membranes (49–57). Depletion of PE and CL affects precursor accumulation at the TOM complex and the activity of the respiratory chain (49, 50 57–59), both of which remain unaffected upon depletion of PC. CL stabilizes respiratory chain supercomplexes and the TOM complex (49, 60–61). In contrast, respiratory chain supercomplexes and the TOM complex are formed in mitochondria with reduced content of either PE or PC (50, 57, 75, 80). The dynamic TIM23 and SAM complexes are particularly sensitive toward alterations of the phospholipid composition of the mitochondrial membranes. The TIM23 translocase is not destabilized when PE levels are reduced (57), whereas depletion of PC or CL affects the integrity of the TIM23 forms on blue native gels. Whether impaired activity of the TIM23 complex contributes to defective protein import into and across the inner membrane in PE-deficient mitochondria remains unclear. In these mitochondria, the membrane potential is strongly reduced, which blocks protein transport via inner membrane protein translocases (57). Import of β-barrel proteins into the mitochondrial outer membrane requires the presence of CL, PE and PC (49, 50, 75). However, the SAM complex is destabilized in mutant mitochondria impaired in PC or CL but not in PE biosynthesis. Furthermore, the SAM-dependent assembly of Tom22 requires normal PC levels (75) but is unaffected in CL- or PE-deficient mitochondria. All these examples illustrate that phospholipids exhibit surprisingly specific roles for distinct protein translocases and respiratory chain complexes in mitochondria.

Experimental Procedures

Yeast Strains, Growth Conditions, and Isolation of Mitochondria—The yeast strains *pem1*Δ, *pem2*Δ, *psd1*Δ and their corresponding wild-type BY4741 have been described (75). The cells were grown at 30 °C on minimal medium containing a non-fermentable carbon source (0.67% (w/v) yeast nitrogen base without amino acids (Difco), 0,077% (w/v) SC amino acid mixture (MP Biomedicals), 2% (v/v) glycerol or lactate and 0.1–0.2% (w/v) glucose). Cells were harvested at mid-logarithmic growth phase, and mitochondria were isolated by differential centrifugation as described (89). In brief, the cell wall was disrupted by incubation with 0.1 M Tris/HCl, pH 9.4, 10 mM DTT and subsequent treatment with zymolyase (Nacalai Tesque) in 1.2 M sorbitol, 20 mM KP_i, pH 7.2. The generated spheroplasts were resuspended in homogenizing buffer (0.6 M sorbitol, 10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1 mM PMSF, and 0.2% (w/v) BSA) and disintegrated by using a Dounce homogenizer. Subsequently, cell debris was removed by centrifugation, and mitochondria were isolated by differential centrifugation. Mitochondrial protein concentration was adjusted to 10 mg/ml in washing buffer (10 mM MOPS/KOH, pH 7.2, 1 mM EDTA, 250 mM sucrose). Subsequently, mitochondria were shock frozen in liquid nitrogen and stored at −80 °C until use.

Protein Import into Isolated Mitochondria and Blue Native Electrophoresis—Radiolabeled precursor proteins were synthesized in a cell-free translation system based on reticulocyte lysate (TnT, Promega) in the presence of [^{35}S]methionine. For the import reaction, isolated mitochondria were incubated with the radiolabeled precursor protein in import buffer (3% (w/v) BSA, 250 mM sucrose, 5 mM methionine, 80 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS/KOH, pH 7.2, 10 mM KH_2PO_4) containing 2 mM ATP, 2 mM NADH, 5 mM creatine phosphate, and 0.1 mg/ml creatine kinase. The import reaction was stopped by transfer on ice and by addition of 8 μM (final concentration) antimycin, 1 μM valinomycin, and 20 μM oligomycin to dissipate the membrane potential. In control reactions, the membrane potential was dissipated before the import reaction was started. Non-imported proteins were removed by treatment with proteinase K (50 μg/ml) for 15 min on ice. The protease was inactivated by an excess amount of PMSF. Mitochondria were reisolated and washed with washing buffer. The mitochondrial pellet was solubilized in Laemmli buffer, and proteins were separated by SDS-PAGE. The import of carrier proteins was studied by blue native electrophoresis. To this end, mitochondria were reisolated after the import reaction, washed with washing buffer, resuspended in lysis buffer (20 mM Tris/HCl, pH 7.4, 50 mM NaCl, 0.1 mM EDTA, 10% (v/v) glycerol) containing 1% (w/v) digitonin, and incubated for 15 min on ice. The sample was subjected to centrifugation to remove insoluble material. Protein complexes were separated by blue native electrophoresis (90).

Arrest of Precursor Proteins—To arrest precursors of cytochrome b_2-DHFR, AAC-DHFR, and Om45-DHFR in the TOM complex, import reactions were performed as described above. The stable folding of the DHFR domain was induced by the presence of 5 μM methotrexate (10, 11). Precursor of Oxa1 was accumulated in the TOM complex upon dissipation of the membrane potential (57, 75).

In-gel Activity Assays—The activity of the F_1F_0-ATP synthase was determined by in-gel activity assays (91, 92). Mitochondrial protein complexes were separated by blue native electrophoresis. The blue native gel was washed in ATP buffer (5 mM $MgCl_2$, 50 mM glycine, pH 8.4, 20 mM ATP) for 20 min at room temperature. Subsequently, the gel was incubated with 10% (w/v) $CaCl_2$ solution until the CaP_i precipitates appeared. The activity of the cytochrome *c* oxidase was determined by in-gel activity stain (79). Protein complexes were separated by blue native electrophoresis. The gel was washed with 50 mM KP_i, pH 7.2. Subsequently, the gel was incubated with 1 mg/ml reduced horse cytochrome *c* in 50 mM KP_i, pH 7.2, in the presence of 1 mg/ml diaminobenzidine to stain active cytochrome *c* oxidase.

Measurement of the Membrane Potential—To determine the membrane potential across the inner mitochondrial membrane, isolated mitochondria were incubated with the fluorescent dye 3,3-dipropylthiadicarbocyanine iodide ($DISC_3$) in potential buffer (0.6 M sorbitol, 0.1% (w/v) BSA, 10 mM $MgCl_2$, 0.5 mM EDTA, 20 mM KP_i, pH 7.2) supplemented with 5 mM succinate and 5 mM malate (55, 57). $DISC_3$ is taken up by mitochondria in a membrane potential-dependent manner, which results in quenching of the fluorescence signal at 670 nm (52). To determine the specificity of the measured signals, valinomycin was added to 1 μM final concentration, which dissipates the membrane potential leading to a release of $DISC_3$ from mitochondria. All measurements were performed with an Aminco

Bowman II luminescence spectrometer (Thermo Electron) using a Hellma-101.OS cuvette and the AB2 software (Thermo Electron).

Phospholipid Analysis—Extraction of phospholipids from total cell extracts and isolated mitochondria was performed with a 2:1 (v/v) mixture of chloroform/methanol as described (37, 75, 93). The lipids in the organic phase were washed with 0.034% (w/v) $MgCl_2$, a 4:1 (v/v) mixture of 2 N KCl/methanol, and subsequently with a mixture of methanol/water/chloroform (48:47:3, per volume). Two-dimensional thin layer chromatography was used to separate the individual phospholipid classes (94). Finally, phospholipids were stained with iodine vapor, scraped off, and quantified (95).

Miscellaneous—For the detection of mitochondrial proteins, we used a large set of polyclonal antisera. To exclude cross-reactions, all immunosignals were validated with mitochondria isolated from the corresponding mutant strains (57, 96). Proteins were transferred from SDS-PAGE and blue native gels onto a PVDF membrane (EMD Millipore) via semi-dry Western blotting. Immunosignals were detected by enhanced chemiluminescence (97) and visualized on x-ray films (Medix XBU) or via the LAS3000 image reader (FujiFilm). Radiolabeled proteins were visualized by autoradiography (Storm Imaging System, GE Healthcare and FLA-9000, FujiFilm). We used the ImageJ version 1,46r (National Institutes of Health) software to analyze autoradiograms. Images were processed with Photoshop CS5 (Adobe) and arranged in figures using Illustrator CS5 (Adobe). Where indicated by separating white lines, non-relevant bands were digitally removed. Quantifications were performed with the ImageQuant version 5.2 (GE Healthcare) software. We used Excel version 14.6.5 to prepare the charts of the quantifications.

Author Contributions—T. B. conceived and coordinated the study and wrote the manuscript. M. H. S., F. D. B., C. U. M., and T. B. designed, performed, and analyzed the experiments together with G. D. M. H. S. and T. B. prepared the figures. All authors reviewed results and approved the final version of the manuscript.

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