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Lipid Conversion by Cell-Free Synthesized Phospholipid Methyltransferase Opi3 in Defined Nanodisc Membranes Supports an *in Trans* Mechanism

Erik Henrich,[†] Frank Löhr,[†] Grzegorz Pawlik,[‡] Oliver Peetz,[§] Volker Dötsch,[†] Nina Morgner,[§] Anton I. de Kroon,*[,‡] and Frank Bernhard*[,†]

[†]Institute of Biophysical Chemistry, Centre for Biomolecular Magnetic Resonance, J.W.-Goethe-University, 60438 Frankfurt am Main, Germany

[‡]Membrane Biochemistry & Biophysics, Bijvoet Center for Biomolecular Research and Institute of Biomembranes, Utrecht University, 3584 CH Utrecht, The Netherlands

[§]Institute of Physical and Theoretical Chemistry, J.W.-Goethe-University, 60438 Frankfurt am Main, Germany

Supporting Information

ABSTRACT: Biomembranes composed of lipids and proteins play central roles in physiological processes, and the precise balance between different lipid species is crucial for maintaining membrane function. One pathway for the biosynthesis of the abundant lipid phosphatidylcholine in eukaryotes involves a membrane-integrated phospholipid methyltransferase named Opi3 in yeast. A still unanswered question is whether Opi3 can catalyze phosphatidylcholine synthesis *in trans*, at membrane contact sites. While evidence for this activity was obtained from studies with complex *in vitro*-reconstituted systems based on endoplasmic reticulum membranes, isolated and purified Opi3 could not be analyzed. We present new insights into Opi3 activity by characterizing the *in vitro*-synthesized enzyme in defined hydrophobic environments. *Saccharomyces cerevisiae* Opi3 was cell-free synthesized and either solubilized in detergent micelles or co-translationally inserted into preformed nanodisc membranes of different lipid compositions. While detergent-solubilized Opi3 was inactive, the enzyme inserted into nanodisc membranes showed activity and stayed monomeric as revealed by native mass spectrometry. The methylation of its lipid substrate dioleoylphosphatidylmonomethylethanolamine to phosphatidylcholine was monitored by one-dimensional ^{31}P nuclear magnetic resonance. Phosphatidylcholine formation was observed not only in nanodiscs containing inserted Opi3 but also in nanodiscs devoid of the enzyme containing the lipid substrate. This result gives a clear indication for *in trans* catalysis by Opi3; i.e., it acts on the substrate in juxtaposed membranes, while *in cis* lipid conversion may also contribute. Our established system for the characterization of pure Opi3 in defined lipid environments may be applicable to other lipid biosynthetic enzymes and help in understanding the subcellular organization of lipid synthesis.

Besides controlling cellular shape, transport of molecules, and communication with the surrounding environment, biomembranes are essential for the subcellular organization of eukaryotic cells. Distinct membrane lipids are involved in numerous cellular processes like signal transduction and apoptosis.[1,2] The lipid composition of membranes can thus act as a central modulator of cell-type or organelle specific functions. Lipids differ in length and flexibility of fatty acid chain moieties as well as in headgroup chemistry, and their biosynthesis is controlled by various enzyme machineries.[3] In *Saccharomyces cerevisiae*, approximately 50% of the membrane lipids belong to the class of phosphatidylcholines (PCs). PC lipid biosynthesis proceeds by two alternative pathways based on either diacylglycerol (Kennedy pathway) or cytidine-diphosphate (CDP)-diacylglycerol (methylation pathway).[4] The latter pathway involves endoplasmic reticulum (ER) membrane-integrated enzymes Cho2 and Opi3 converting phosphatidylethanolamine (PE) to phosphatidylmonomethylethanolamine (PMME) and PMME via phosphatidyldimethylethanolamine (PDME) to PC, respectively.[5] *S*-Adenosylmethionine (SAM) is required by both enzymes as a methyl donor. Lipid conversion by the two enzymes affects cellular processes including mitophagy.[6] Biochemical as well as genetic studies provided evidence for an *in trans* catalysis mechanism of Opi3 by converting lipids in a juxtaposed membrane at membrane contact sites.[7,8] Such membrane contact sites between the ER and the plasma membrane[9] have been proposed as major hubs of lipid exchange[10] and to play a role in regulating cellular lipid homeostasis.[11] The mechanism of *in trans* lipid synthesis[8,12] modifies the overall understanding of lipid metabolism as extensive lipid trafficking would become dispensable. Membrane contact sites could facilitate the close contact between enzymes and lipids from different membranes to enable *in trans* catalysis of lipid conversion, as was recently shown for phosphatidylserine decarboxylase located in the inner mitochondrial membrane that converts substrate in the outer membrane.[13] However, studies with purified Opi3 in defined membranes could not be performed, and its mode of

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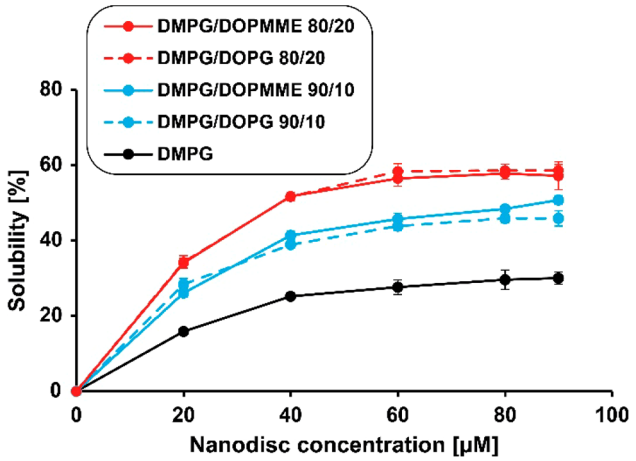

Figure 1. Insertion of Opi3 into different nanodisc membranes. The Opi3−sfGFP construct was cell-free synthesized in the presence of increasing concentrations of supplied preformed nanodiscs. Membrane insertion of the Opi3−sfGFP construct was correlated with folding of the sfGFP moiety and monitored by sfGFP fluorescence. Lipid compositions of the nanodisc membranes are given in the inset. Data were generated by three independent experiments, and the error bars depict the standard deviation.

action is therefore still under debate. Hence, further molecular details about Opi3 function are crucial for understanding mechanisms of lipid conversion.

Within the past decade, cell-free expression systems became a powerful tool for producing challenging membrane proteins.[14] Even detergent sensitive membrane proteins can efficiently be synthesized by co-translational insertion into defined membranes of preformed nanodiscs.[15,16] The reduced complexity of cell-free systems further improves downstream protein purification and characterization processes.[17] In addition, the lack of any phosphatidylethanolamine methyltransferase activity in *Escherichia coli*[18] further facilitates the study of Opi3-like enzymes in *E. coli*-based cell-free systems.

Yeast Opi3 was first cell-free synthesized in the absence of any supplied nanodiscs, and the expression efficiency optimized at a final Mg^{2+} ion concentration in the reaction mixture between 16 and 18 mM was 0.7−1 mg/mL (Figure S1a). The protein precipitated and was subsequently solubilized in the detergent dodecyl β-D-maltoside (DDM) or 1-myristoyl-2-hydroxy-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (LMPG). Both detergents were previously identified as being suitable for the solubilization of several cell-free generated protein precipitates.[19] While DDM was inefficient, the harsher detergent LMPG completely solubilized the Opi3 precipitates. However, LMPG-solubilized Opi3 did not show enzymatic activity (data not shown). Improper folding after solubilization of precipitated Opi3 and/or sensitivity toward contact with detergents might thus have inactivated the enzyme.[20] We therefore tried next to insert the enzyme co-translationally into preformed empty nanodisc membranes supplied to the cell-free reaction.[21] Nanodiscs were assembled with the scaffold protein MSP1E3D1 and a variety of different lipids and lipid mixtures, including dimyristoylphosphatidylglycerol (DMPG), dioleoylphosphatidylglycerol (DOPG), and the Opi3 methylation substrate dioleoylphosphatidylmonomethylethanolamine (DOPMME) (Table S1). These lipids have previously been shown to support the co-translational integration of even complex membrane proteins.[15,16] Unfortunately, assembly mixtures containing the lipid dimyristoylphosphatidylcholine (DMPC), a representative of the most abundant phospholipid class in eukaryotes, in combination with 10−30 mol % DOPMME failed to produce homogeneous disc populations (Figure S2). The solubilization of a cell-free synthesized Opi3−sfGFP construct was analyzed in the presence of increasing concentrations of DMPG nanodiscs (Figure 1). The sfGFP moiety folds only if the Opi3−sfGFP construct remains soluble by insertion into the nanodiscs, and the sfGFP fluorescence was therefore taken as a measure of membrane insertion of the Opi3−sfGFP construct.[16,21] Increased concentrations of DOPMME and DOPG in the membrane improved the solubilization of the Opi3−sfGFP

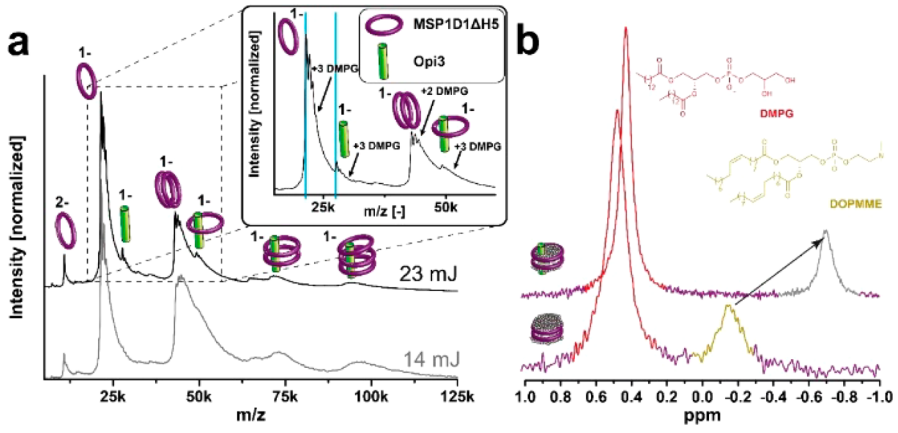

Figure 2. Oligomeric state of Opi3 and lipid conversion in nanodiscs. (a) LILBID mass spectrometry analysis of Opi3 in nanodiscs. Pictograms illustrate detected species (see the legend), and signals corresponding to attached lipids are indicated in the inset, where the blue lines highlight the theoretical masses of MSP1D1ΔH5 and the Opi3 monomer. Detailed numbers of theoretical and experimentally detected masses are listed in Table S2. Laser powers used in the LILBID setup are given in millijoules. (b) One-dimensional NMR spectra detecting the signal of the ^{31}P atoms of the phosphate moiety in the lipid headgroup were recorded on a 500 MHz Bruker DRX spectrometer. Nanodiscs formed with the scaffold protein MSP1D1ΔH5 and with a DMPG/DOPMME ratio of 80/20 were analyzed before (bottom) and after (top) insertion of Opi3. Chemical structures of the lipids are displayed, and the peaks are highlighted in the colors corresponding to the colored structures. Pictograms illustrate the type of analyzed complex. The arrow in panel b illustrates the shift of the DOPMME signal after Opi3 insertion.

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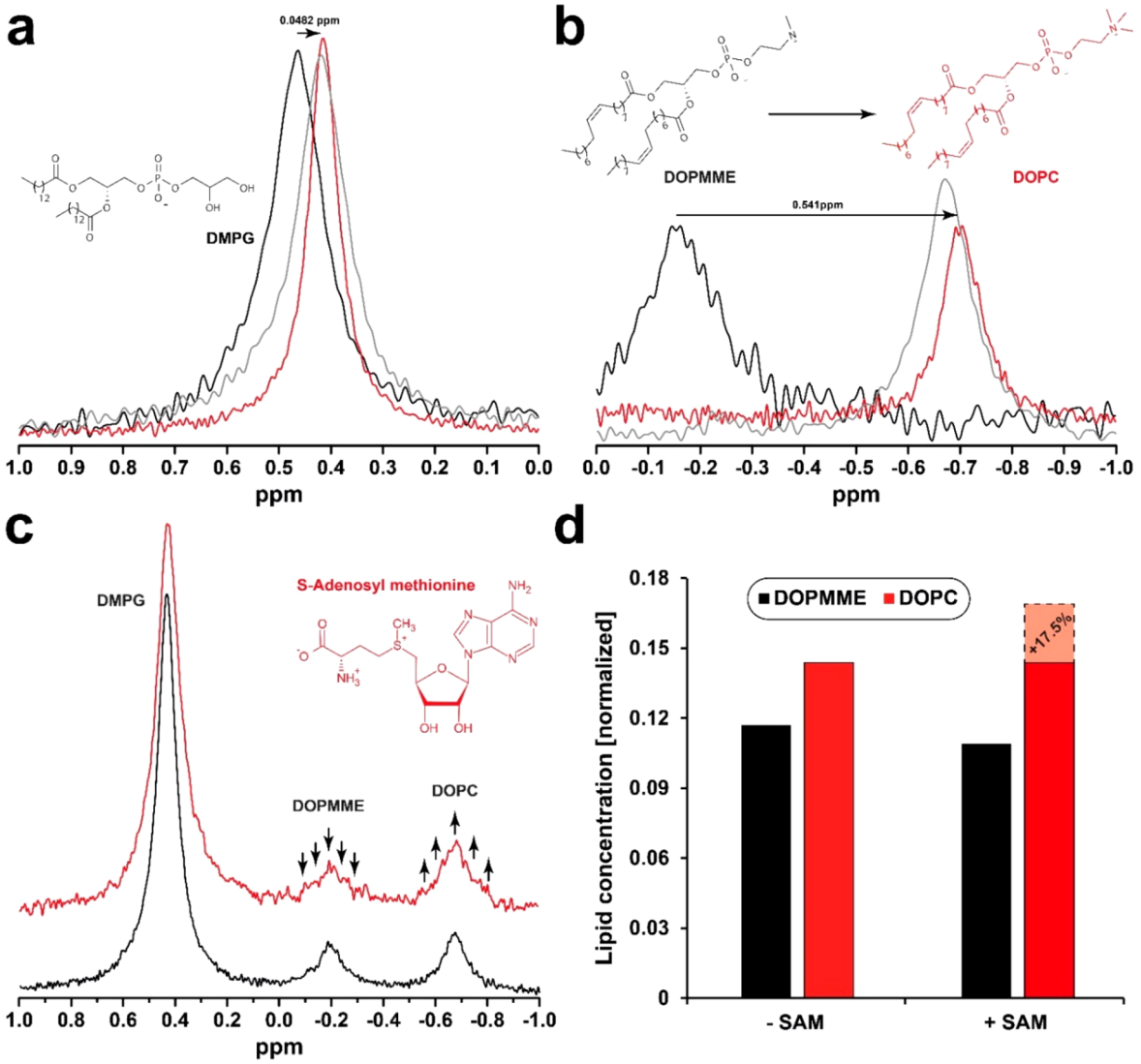

Figure 3. Lipid conversion by Opi3. In panels a (DMPG region) and b (PE and PC region), overlays of ^{31}P NMR spectra are depicted to demonstrate the enzymatic conversion of DOPMME to DOPC by Opi3. The spectra of empty DMPG/DOPMME nanodiscs (black), Opi3-loaded nanodiscs (red), and 50% DMPG/50% DMPC nanodiscs (gray) are given. Chemical structures of lipids are shown, and the signal shifts are indicated by black arrows. (c) Lipid conversion in empty nanodiscs. Empty nanodiscs (80 μM, 80/20 DMPG/DOPMME) were added to approximately 10−15 μM purified Opi3 nanodiscs (DMPG/DOPC) giving three ^{31}P signals corresponding to DMPG, DOPMME, and DOPC (black). The supply of 5 mM SAM (incubation for 10 min at room temperature) results in further conversion of DOPMME to DOPC (red curve) as highlighted by black arrows. The decreased signal-to-noise ratio is attributed to the partial denaturation of the nanodiscs due to the low pH of the SAM stock solution and may cause larger variations in peak integration. (d) Quantification of lipid concentrations. The peaks in the NMR spectra in panel c were integrated, and the relative concentrations of DOPMME and DOPC, normalized to the peak of DMPG as a reference, before and after the addition of SAM are plotted in a bar diagram. The ∼17.5% increase in the level of DOPC after SAM addition is highlighted by a box with a dashed line.

construct (Figure 1). The higher flexibility of DOPG in combination with the larger acyl chain length might thus support the membrane insertion of Opi3,[22] while binding of its substrate DOPMME could further stabilize the protein.

Many membrane proteins form larger homo-oligomeric complexes in membranes or may even exist in multiprotein assemblies.[23,24] We therefore implemented native laser-induced-liquid-bead-ion-desorption (LILBID) mass spectrometry as an ideal tool to analyze membrane protein oligomerization in nanodisc membranes.[23] Corresponding spectra of Opi3 inserted into nanodisc membranes composed of DMPG and DOPMME at an 80/20 ratio revealed only monomeric species of the enzyme (Figure 2a).

We next used one-dimensional ^{31}P nuclear magnetic resonance (NMR) to analyze the functional activity of Opi3. First, various nanodiscs comprising different engineered scaffold proteins[25] with resulting disc sizes from 8 to 12 nm combined with lipids or lipid mixtures were prepared and analyzed by NMR through detection of ^{31}P signals from the lipid headgroup (Figure S3). Signals of individual lipid types in membranes containing DMPC, DMPG, and/or DOPMME could be identified and quantified by this technique (Figure S3). Nanodiscs assembled with the small scaffold protein MSP1D1ΔH5 giving the best NMR signals were selected for further experiments (Figure S4). These nanodiscs were added to cell-free reaction mixtures and purified from the reaction mixture after expression and insertion of Opi3. The

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purification was performed via the C-terminal His-tags of Opi3 and of the MSP1D1ΔH5 scaffold protein (Figure S1b,c). Consequently, the purified samples contained nanodiscs with inserted Opi3 as well as empty nanodiscs.

We analyzed nanodiscs assembled with 80% DMPG and 20% DOPMME before and after insertion of Opi3. While the intensities of the lipid signals did not change significantly, the signal corresponding to DOPMME shifts to a higher field (Figure 2b).

The reason for the DOPMME shift could be either the change of the chemical environment upon binding to Opi3 or the conversion of DOPMME to DOPDME or DOPC already during the cell-free reaction and even in the absence of the supplied SAM substrate. Via comparison of the lipid signal positions from DMPG/DOPMME nanodiscs before and after Opi3 insertion with that of a DMPG/DMPC reference sample, it is evident that the DMPG signals match with each other (Figure 3a), while the signal originating from DOPMME now overlaps with the signal of DMPC (Figure 3b). This is a clear indication that DOPMME was converted to DOPC already during cell-free expression. *E. coli* amino acid metabolism pathways converting methionine to cysteine produce SAM as a first intermediate (Figure S5). This reaction is catalyzed by *S*-adenosylmethionine synthase, an enzyme that is still present in the *E. coli* S30 lysates used for cell-free synthesis as revealed by recent proteomics studies.[17] The SAM methyl donor that is essential for the conversion of DOPMME to DOPC by Opi3 is thus already present in the cell-free reaction mixture and in combination with the mimicked cytosolic milieu of the reaction mixture and the incubation temperature of 30 °C generates a suitable reaction environment for Opi3.

The concentration of Opi3−sfGFP/MSP1D1ΔH5 discs in the cell-free reaction mixture was estimated to be approximately 15 μM according to the sfGFP fluorescence. As the total concentration of supplied nanodiscs is 60 μM, 75% of the nanodiscs remain empty. It is thus interesting to note the apparently quantitative conversion of DOPMME to DOPC in Opi3-containing nanodiscs as well as in empty nanodiscs (Figure 2b). This indicates that Opi3 can convert lipids *in trans* in juxtaposed membranes, as fusion of nanodiscs or extensive lipid exchange seems to be unlikely.[26] To further verify this assumption, 80 μM empty MSP1D1ΔH5 nanodiscs containing DMPG with 20% DOPMME lipids were added to an approximately 10−15 μM purified Opi3 nanodisc sample. After the reaction had been started by the addition of SAM and a 10 min incubation at room temperature, an ∼17% increase in the level of DOPC along with the consumption of DOPMME was detected by ^{31}P NMR (Figure 3c,d).

Our findings agree with previous studies based on crude microsome preparations, suggesting *in trans* lipid conversion by Opi3.[7,8] However, these lack controls for the occurrence of lipid exchange and/or membrane fusion. The wild-type microsomes used[8] harbor Cho2, the phosphatidylethanolamine methyltransferase that might contribute to the methylation of PMME, and may contain proteins capable of transferring lipids between membranes. This study based on purified Opi3 inserted into defined and stable nanodisc membranes demonstrated *in trans* lipid conversion activity in a controlled system, while additional *in cis* lipid conversion cannot be excluded. The results further support the proposed importance of membrane contact sites between different organelles for lipid biosynthesis.[27] While these complexes are expected to facilitate the exchange of lipids, the proximity of membranes from different organelles could also promote the *in trans* conversion of lipids. In the artificial environment of cell-free reactions, an incubation time of approximately 12 h was obviously sufficient for the complete *in trans* conversion of DOPMME to DOPC in supplied nanodiscs. The demonstration of efficient *in trans* lipid conversion activity by Opi3 could modify the current view of cellular lipid biosynthesis, as extensive lipid trafficking mechanisms between organelles are obviously not absolutely mandatory.[3,27] Future studies focusing on the mechanistic details of other Opi3-related membrane integrated enzymes could thus be valuable for completing a comprehensive picture of lipid biosynthesis and trafficking in cells.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.8b00807.

Detailed text description of methods and figures as well as tables relating to sample preparation (Table S1 and Figures S1, S2, and S4), masses of proteins and complexes (Table S2), NMR data (Figure S3), and a description of a biochemical pathway (Figure S5) (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: A.I.P.M.deKroon@uu.nl.
*E-mail: fbern@bpc.uni-frankfurt.de.

ORCID

Nina Morgner: 0000-0002-1872-490X
Frank Bernhard: 0000-0002-4860-1783

Author Contributions

E.H., A.I.d.K., and F.B. conceptualized the project. E.H., F.L., G.P., and O.P. conducted experiments. E.H. and F.B. wrote the manuscript with the participation of all authors.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CDP, cytidine diphosphate; DDM, dodecyl β-D-maltoside; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DO, dioleoyl; DOPG, dioleoylphosphatidylglycerol; ER, endoplasmic reticulum; LILBID, native laser-induced-liquid-bead-ion-desorption; LMPG, 1-myristoyl-2-hydroxy-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PDME, phosphatidyldimethylethanolamine; PE, phosphatidylethanolamine;

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Biochemistry 2018, 57, 5780−5784

PMME, phosphatidylmonomethylethanolamine; SAM, *S*-adenosylmethionine.

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