Cell Chemical Biology

Structural Lipids Enable the Formation of Functional Oligomers of the Eukaryotic Purine Symporter UapA

Graphical Abstract

Highlights

- Mass spectrometry reveals specific lipid binding to the eukaryotic transporter UapA
- Interfacial lipids stabilize the functional UapA dimer
- MD simulations reveal the lipid binding sites
- Mutagenesis of a lipid binding site disrupts UapA dimerization and function in vivo

Authors

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In Brief

We describe the first in-depth analysis of membrane lipid interactions with a eukaryotic transporter using native mass spectrometry. We demonstrate that the binding of structural lipids is essential to maintain the stability of the functional UapA dimer in both the gas phase and in vivo.
Structural Lipids Enable the Formation of Functional Oligomers of the Eukaryotic Purine Symporter UapA

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SUMMARY

The role of membrane lipids in modulating eukaryotic transporter assembly and function remains unclear. We investigated the effect of membrane lipids in the structure and transport activity of the purine transporter UapA from Aspergillus nidulans. We found that UapA exists mainly as a dimer and that two lipid molecules bind per UapA dimer. We identified three phospholipid classes that co-purified with UapA: phosphatidylcholine, phosphatidylethanolamine (PE), and phosphatidylinositol (PI). UapA delipidation caused dissociation of the dimer into monomers. Subsequent addition of PI or PE rescued the UapA dimer and allowed recovery of bound lipids, suggesting a central role of these lipids in stabilizing the dimer. Molecular dynamics simulations predicted a lipid binding site near the UapA dimer interface. Mutational analyses established that lipid binding at this site is essential for formation of functional UapA dimers. We propose that structural lipids have a central role in the formation of functional, dimeric UapA.

INTRODUCTION

Cellular membranes play key roles in determining the structure and function of membrane proteins (Opekarova and Tanner, 2003). Membranes are highly fluid and asymmetrical structures that provide distinct physical environments for the associated proteins (Engelman, 2005). The biophysical properties of the membrane, such as lateral and transverse pressures caused by membrane curvature and lipid packing, directly affect membrane protein folding, structure, and function (Booth and Curnow, 2009; Marsh, 1996; van den Brink-van der Laan et al., 2004). Furthermore, both specific and non-specific protein-lipid interactions can affect transporter conformation, stability, and oligomerization (Gupta et al., 2017; Koshy and Ziegler, 2015; Laganowsky et al., 2014; Martens et al., 2016). For example, specific lipid binding to subunit-subunit interfaces can modulate BetP oligomerization (Koshy et al., 2013). In contrast, bulk or annular lipid interactions can provide structural support to facilitate conformational changes in the transporter NapA by stabilizing the position of a static gate domain while allowing the movement of dynamic core domain to accommodate an elevator-like mechanism (Landreh et al., 2017). Therefore, it is essential to characterize protein-lipid interactions and the relationship between proteins and the cell membrane to fully understand the structure and function of membrane proteins in vivo.

Traditional structural biology methods, such as cryoelectron microscopy and X-ray crystallography, have offered invaluable information regarding membrane protein structure and function; however, even with these high-resolution methods capturing protein-lipid interactions remains a challenge (Peng et al., 2014). While there has been a marked increase in the number of membrane protein structures with resolved lipids in recent years, only a select few studies have established the structure-function relationship of protein-lipid interactions (Koshy et al., 2013; Mehmood et al., 2016; Norimatsu et al., 2017). Mass spectrometry (MS) of intact protein complexes has emerged as a key method for detecting, identifying, and characterizing protein-lipid interactions (Gupta et al., 2017; Henrich et al., 2017; Laganowsky et al., 2013; Landreh et al., 2017; Mehmood et al., 2016; Reading et al., 2015; Skruzny et al., 2015; Zhou et al., 2011). Native MS employs nano-electrospray ionization as a “soft” ionization technique that preserves the structure, oligomerization, and ligand-lipid interactions of protein complexes (Ahdash et al., 2017; Ashcroft, 2005; Hernandez and Robinson, 2007). During native MS of intact membrane proteins, detergent micelles are removed through collisional activation with inert gas molecules. Careful tuning of the instrument parameters ensures well-resolved spectra, while maintaining the overall protein fold as well as interactions with other proteins, ligands, and/or lipids. Consequently, native MS has emerged as a powerful tool for examining the role of lipid binding on the stability of membrane

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protein oligomers (Gupta et al., 2017; Reading et al., 2015; Wang et al., 2010).

Recent studies using native MS have demonstrated that protein-lipid interactions play a crucial role in stabilizing the dimer form of prokaryotic transporters (Gupta et al., 2017; Henrich et al., 2017). However, our understanding of the role of lipids in maintaining the quaternary structure of eukaryotic transporters remains limited, mainly due to the relatively poor stability of these proteins in non-native environments. Here, we used native MS to study UapA, a eukaryotic transporter from *Aspergillus nidulans* belonging to the nucleobase ascorbate transporter (NAT) family of metabolite importers. UapA is responsible for H⁺-dependent uptake of the purines xanthine and uric acid. The high-resolution structure of a transport inactive, conformationally locked mutant of UapA (G411VΔ1-11) was recently solved, revealing that UapA is formed from two domains, the core domain and gate domain, and is likely to transport via an elevator mechanism (Alguel et al., 2016). The structure also showed that UapA is a homodimer confirming earlier biochemical studies (Martzoukou et al., 2015). Analysis of the structure in combination with mutagenesis data demonstrated that dimer formation was essential for function (Alguel et al., 2016). The structure showed that the extensive (~6,000 Å²) dimer interface was mainly mediated by hydrophobic interactions and displayed no electron density consistent with lipid binding (Alguel et al., 2016). We combined native MS with molecular dynamics (MD), mutagenesis, and functional analyses to establish that phosphatidylinositol (PI) and phosphatidylethanolamine (PE) are closely associated with UapA and play a crucial role in stabilizing the functional UapA dimer.

RESULTS

Protein-Lipid Interactions Stabilize the UapA Dimer

We began by subjecting a purified thermostabilized, conformationally locked, inward-facing mutant of UapA (G411VΔ1-11) (MW = 60,859.59 Da) (STAR Methods and Figure S1A) to ion-mobility (IM)-MS (Leung et al., 2013). IM-MS reports the molecular shape of biomolecules by determining their rotationally averaged collisional cross-section (CCS) (Jennner et al., 2011; Jurneczko and Barran, 2011; Konjinenberg et al., 2014; Michaelevski et al., 2010; Ruotolo et al., 2009). We found the experimental CCS of UapAG411VΔ1-11 (6,117 Å) to be in agreement with the theoretical CCS of the native-like state, calculated from the
crystal structure of UapAG411V

Next, to investigate the oligomeric states of UapA, we performed native MS on UapAG411V_1-11. Well-resolved spectra showed that the protein exists both as a monomer and a dimer (Figures 1A and 1B). The relative abundance of monomer/dimer was ~5:95, suggesting a strong dimer interface (Yefremova et al., 2017). The spectra also revealed the binding of two adduct molecules (measured mass: 775 ± 50 Da) to the UapAG411V_1-11 dimer. We attributed this to the presence of bound phospholipids; however, the resolution of the mass spectra was insufficient to allow identification of the lipid species. We therefore extracted the lipids from the purified protein (LC-MS) and tandem MS (LC-MS/MS) experiments. We identified phosphatidylcholine (PC), PE, and PI as the major lipid classes. We therefore extracted the lipids from the purified protein and analyzed the lipid extract by liquid chromatography-MS (LC-MS) and tandem MS (LC-MS/MS) experiments. We identified phosphatidylcholine (PC), PE, and PI as the major lipid classes. We further confirmed almost total removal of lipid from the samples (Figure S3A). To explore the effect of the different lipids identified in lipidomics on UapA dimerization, we added PI, PE, or PC individually, or in combination, into delipidated UapAG411V_1-11 at a ratio of 1:100 protein/lipid (Figure 2). Addition of either PE or PI restored the UapA dimer with both lipids displaying similar levels of dimer recovery, yielding 73.1% and 59.7% dimer, respectively (Figure 2). The addition of an equimolar mixture of PI and PE to delipidated UapAG411V_1-11 was more efficient at dimer recovery, yielding 81.5% dimer (Figure S3C). This implies that the effects of the two lipids are additive. As a negative control we also titrated phosphatidylglycerol (PG), a lipid not usually found in eukaryotic plasma membranes, into delipidated UapAG411V_1-11 (Opekarska and Tanner, 2003). PG did not induce any significant changes in the oligomerization of delipidated UapAG411V_1-11 (Figure S3B).

In contrast to PI and PE, the addition of PC to delipidated UapAG411V_1-11 failed to mediate the reformation of the UapA dimer. Indeed, adding PC actively induced dissociation of non-dimerized UapAG411V_1-11 (Leung et al., 2013) (Figure S3D). Surprisingly, the presence of PC inhibited the dimer-stabilizing effects of PI and PE when mixtures of the three lipids were added to delipidated UapAG411V_1-11 (Figure S3C). Taken together, these data suggest that the dimer-stabilizing effects are specific to PI and PE lipids.

Next, we investigated the effects of lipid binding on the functional activities of UapA dimers. Indeed, adding PC actively induced dissociation of non-dimerized UapAG411V_1-11 (Figure S3D). Surprisingly, the presence of PC inhibited the dimer-stabilizing effects of PI and PE when mixtures of the three lipids were added to delipidated UapAG411V_1-11 (Figure S3C). Taken together, these data suggest that the dimer-stabilizing effects are specific to PI and PE lipids.

MD Simulations Predict Lipid Binding Sites at the Dimer Interface

MD simulations were performed to predict likely lipid binding sites. We carried out 5-μs simulations using a model of WT...
Mutations to the Putative Lipid Binding Site Cause Loss of UapA Function In Vivo

To explore the role of the putative PI binding site in the structure and function of the UapA dimer, we generated a range of single, double, and triple mutants of the residues (R287A, R478A, R479A, R478A/R479A, R287A/R478A/R479A) with C-terminal GFP tags. We individually transformed these constructs into a uapA strain of A. nidulans pabaA1 argB2 D uapC D azgA D, both the triple R287A/R478A/R479A and double R478A/R479A mutants were unable to grow on either uric acid or xanthine. The single R479A mutant also showed reduced growth on both substrates (Figure 4A).

Fluorescence microscopy carried out at 25°C showed that all of the GFP-tagged UapA mutants localized effectively to the plasma membrane, confirming that loss of transport activity in the mutants is not a result of impaired UapA trafficking (Figure 4B). Further analysis using [3H]xanthine uptake assays (Figures 4C and 4D) confirmed the results from the growth assays and revealed close to WT substrate binding affinity for all mutants. This implies that the R478A/R479A and R287A/R478A/R479A mutations affect substrate transport rather than substrate binding. We have previously shown that dimer formation is critical for UapA function (Alguel et al., 2016), so in order to examine the oligomeric status of the UapA mutants we carried out bimolecular fluorescence complementation (BiFC) analysis using a split YFP system (Martzoukou et al., 2015). This system uses two copies of the individual mutants co-expressed as fusions with either the N- or C-terminal domains of YFP. In this case, fluorescence is observed upon dimerization of copies of UapA tagged with the different YFP domains. Although the R287A/R478A/R479A mutant (RRR/A) was found to traffic to the membrane (Figure 4B), it was much less efficient at reconstituting the functional dimer form. There is evidence of minor lipid

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Figure 3. MD Simulations Predict a Lipid Binding Site at the Dimer Interface of UapA

The simulations were carried out in a symmetric bilayer of PE/PI 40:25:35. Cartoon representation of UapA (A) with one monomer colored blue and the other colored pink. The protein is shown from both sides looking through the membrane (upper panel) and from the intracellular side of the membrane (lower panel). Residues with a normalized contact probability with PI higher than 0.8 over the course of the simulation (Figure S5B) are shown in a space-filling representation in dark blue or dark pink. The region highlighted in the dotted circle indicates those residues predicted to form a lipid binding site at the dimer interface of UapA and is shown in close-up view in (B), where individual amino acid residues are shown in stick representation and labeled with the residue number.
binding peaks to the UapA RRR/A + G411V D 1-11 construct and limited dimer reformation after the addition of PI at a ratio of 1:100 protein/lipid, although at a much lower level than that seen for UapA G411V D 1-11. It is possible that some of the lipid binding we observe is due to PI binding to the alternative binding sites in UapA predicted by our MD simulations. Furthermore, it is also possible that lipid can still associate to the altered binding site at the dimer interface but with markedly reduced affinity, explaining why the protein purifies as a monomer.

**DISCUSSION**

Recent years have seen a growing understanding of the key roles played by membrane lipids in the structure and function of membrane proteins; however, as yet there has been no in-depth study of the effects of lipids on eukaryotic transporters. Here, we explore the roles of lipids in maintaining the functional dimeric form of the eukaryotic UapA transporter. We combine native MS with MD simulations and validate the physiological relevance of their findings using mutagenesis and in vivo functional characterization. Furthermore, it is also possible that lipid can still associate to the altered binding site at the dimer interface but with markedly reduced affinity, explaining why the protein purifies as a monomer.

acquired for this protein are of reduced quality compared with the UapAG411V D 1-11 construct due to the lower stability of the WT protein. However, this gave us confidence that further studies using UapAG411V D 1-11 were representative of the native protein. As mutagenesis of the putative lipid binding site abolished lipid binding, we speculate that PI and PE have defined binding sites and therefore act as so-called structural lipids, forming integral interactions with UapA (Bechara and Robinson, 2015). The dimer interface of UapA has a substantial surface area of ~6,000 A² (Alguel et al., 2016), which should render the dimer very stable; thus, the dissociation of the dimer into the monomeric form upon delipidation may be a result of the comparatively harsh treatment of the molecule rather than a direct effect of associated lipids. However, the fact that the dimer can be reformed by the addition of PI, PE, or a combination of both lipids is strongly suggestive that these lipids have a specific stabilizing effect on the oligomeric form of the transporter. There does not seem to be an absolute requirement for either PI or PE, as individually both can recover dimer from delipidated protein to a similar extent. Interestingly, the addition of both PI and PE was more effective at dimer stabilization than the addition of PI or PE individually, suggesting the effects of these two lipids is additive. MD simulations predict that these lipids bind different regions of the protein. Together, this suggests that the dimer can be further stabilized by interactions at an alternative lipid
binding site. It is also possible that PE binding induces positive allosteric modulation of PI binding; such effects have been reported previously for AmTB (Patrick et al., 2018). Further research will be required to confirm the location of alternative dimer-stabilizing lipid binding sites.

Interestingly, MD simulations predicted that most specific lipid binding sites are found within regions of the protein located on the intracellular side of the membrane and are therefore in contact with lipids in the inner membrane leaflet. Similar predictions have been recently made for NapA (Landreh et al., 2017) and CitS (Wöhler et al., 2015) transporters. A high-resolution crystal structure of BetP also revealed that asymmetry of lipids is important in mediating oligomer formation with a preference for lipids in the inner leaflet (Koshy et al., 2013).

Figure 5. The R287A/R478A/R479A UapA Mutant Reduces Dimer Formation
(A) Bimolecular complementation (BiFC) analysis of the R478A/R479A (RR/A) UapA mutant and the R287A/R478A/R479A (RRR/A) UapA mutant. Mutant constructs tagged with the individual YFP domains were co-expressed in A. nidulans. Upon UapA dimerization the YFP is reconstituted. YFP fluorescence was measured by epifluorescence inverted microscopy. WT UapA (uapA +) expressed individually with either the C-terminal domain of YFP (YFPC) or the N-terminal domain of YFP (YFPN) is the negative control.
(B) Relative quantification of plasma membrane fluorescence intensity of mutants compared with WT UapA +/− YFPC/UapA +/− YFPN.
(C) (Left) Mass spectrum of UapA RRR/A with a G411V mutation and 11-residue N-terminal truncation. (Right) Relative abundances of each oligomer of UapA RRR/A + G411V D1-11.
(D) (Left) Mass spectrum of UapA RRR/A + G411V D1-11 with PI (34:1) added at a ratio of 1:100 protein/lipid. (Right) Relative abundances of each oligomer of UapA RRR/A + G411V D1-11 with PI (34:1) added at a ratio of 1:100 protein/lipid. See Figure 1A for the relative abundances of each oligomer of UapAG411V D1-11 under identical conditions. The relative abundance data are the average ± SD, n = 3.
(E) Schematic showing the effect of the R287/R479/R478 mutations on lipid stabilization of the UapA dimer. Mutations of R287A/R478A/R479A abolish lipid binding capability, resulting in the dissociation of UapA into monomers.
Of the three residues comprising the putative lipid binding site at the UapA dimer interface, only R479 is highly conserved among the eukaryotic NAT proteins. Interestingly, the R479A mutation was the only single substitution that had detectable effects on the A. nidulans growth rate. A loss of function observed for the R478A/R479A double mutant at the physiological temperature for A. nidulans, 25°C, and for the R287A/R478A/R479A triple mutant at both 25°C and 37°C was not due to either a loss of correct trafficking to the membrane or impaired substrate binding. The apparent cryosensitivity of the single (R479A) and double (R478A/R479A) mutants may be due to a change in the A. nidulans lipid composition at the lower temperature. Such temperature-dependent lipid composition changes have been reported for S. cerevisiae (Eising et al., 2009).

Native MS analysis of the purified triple mutant in the G411V background indicated that this construct is almost exclusively monomeric. Given that the functional form of UapA is the dimer, we reason that the lack of transport function in this protein is due to its inability to bind lipids that promote dimerization. However, it is important to note that both native MS and the BiFC assay indicate that dimer does form in the absence of lipid binding, although to a much lower extent than when the lipid binding site at the dimer interface is intact. However, this form of the protein has lost virtually all transport activity. This indicates that lipid binding is not an absolute requirement for dimer formation but is essential for formation of the functional dimer, which suggests that lipid binding could form part of the regulatory mechanism of UapA. This hypothesis could form the basis of future investigations into the nature of UapA-lipid interactions.

The MD simulations predicted that lipids could also bind to the outermost, membrane-facing regions of the core domains of the UapA dimer (see Figure S1D for domain organization of UapA). Lipid binding to this region has been predicted for NhaA and suggested to be involved in stabilizing the core domain during the conformational transitions required for transport by the elevator mechanism (Landreh et al., 2017). However, in the case of UapA, removal of the putative lipid binding site at the dimer interface caused almost total loss of lipid binding as revealed by native MS (Figure 5C). This indicates that binding to the core domain may not be a feature of UapA-lipid interactions. Further research is required to confirm this.

In conclusion, we have performed the first detailed analysis of the role of lipid binding to a eukaryotic transporter UapA, and have shown that specific structural lipids are critical for maintaining the protein in a functional dimeric state. Overall, the combination of approaches used here has clear potential to allow investigation of the function of lipid binding to a range of different membrane proteins.

**Significance**

Lipids play an important role in the stability and function of membrane transporters. It has been proposed that the binding of lipids to specific sites on membrane proteins is integral to both protein structure and function. Here, we have characterized structural lipids associated with a eukaryotic transporter, UapA, using native mass spectrometry and *in vivo* functional analysis. We propose that structural lipids stabilize the dimer interface of UapA and are essential for transport activity. These findings expand our understanding of eukaryotic protein-lipid interactions and have direct implications for the molecular mechanism of UapA transport.

**STAR Methods**

Detailed methods are provided in the online version of this paper and include the following:

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  - Lipid Titrations
  - IM-MS
  - LC-MS
  - Coarse-grained Molecular Dynamics (CG-MD) Simulations
  - *A. nidulans* Growth Conditions, UapA Localization, Bioluminescence Complementation Assays and Xanthine Transport Assays
- **Quantification and Statistical Analysis**

**Supplemental Information**

Supplemental Information includes five figures and one table and can be found with this article online at https://doi.org/10.1016/j.chembiol.2018.03.011.

**Acknowledgments**

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**Author Contributions**

A.P. and B.B. designed the project; E.P. expressed and purified the protein; Z.H. carried out lipidomics experiments; E.P. analyzed the protein by native MS and IM-MS; A.C.K. performed MD simulations; S.A. and G.D. carried out the mutagenesis functional analysis and localization studies of the protein; A.M.L. and A.C.H. analyzed the data; E.P., B.B., and A.P. wrote the manuscript with contributions from all authors.

**Declaration of Interests**

The authors declare no competing interests.
REFERENCES


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STAR METHODS

KEY RESOURCES TABLE

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Experimental Models: Organisms/Strains

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Software and Algorithms

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Argyris Politis (argyris.politis@kcl.ac.uk).

METHOD DETAILS

Expression of the UapA Constructs and Membrane Preparation

Wild-type (WT) UapA, a thermostable construct of UapA (UapAG411VΔ1-11), and a construct of UapA with disrupted lipid binding (R287A/R478A/R479A with G411VΔ1-11) (RRR/A + G411VΔ1-11) were recombinantly expressed as described previously (Alguel et al., 2016). In brief, S. cerevisiae FGY217 cells (6 or 12L) containing one of the UapA constructs were grown at 30°C and shaking at 300 rpm to an OD600 of 0.6. Expression was induced by the addition of galactose to a final concentration of 2%. After 22 hr incubation at 30°C with shaking the cells were harvested by centrifugation and resuspended in 10 mL cell resuspension buffer (50 mM...
The relative abundances of each oligomeric state and lipid bound state of UapA was calculated using UniDec, a spectrum deconvolution software package (Marty et al., 2015), after correcting peak intensity by accounting for detector efficiency (Fraser, 2002). Spectra were smoothed using Masslynx 4.1 (Waters) software prior to deconvolution. The mass range for peak detection was 50000-126000 Da. The following parameters were adjusted between samples to minimise the assignment of background noise as UapA species: charge (minimum of 8, maximum of 32), mass (monomer: 61025 ± 25, dimer 122025 ± 25, dimer + lipid 123550 ± 50 Da), and intensity threshold (0.00 to 0.45). This method assumes all UapA species have similar ionisation efficiencies.
IM-MS

Conditions in the mass spectrometer for IM-MS of the dimer were: capillary voltage +1.2-1.6 kV, sampling cone voltage 5 V, trap CE 20 V, transfer CE 200 V, capillary backing pressure 0 bar. Drift times were measured at a T-wave height of 40 V and at three T-wave velocities (550, 600, 640 m/s). The following range of calibrants were utilised at 10 μM in 200 mM ammonium acetate: concanavalin A, β-lactoglobulin, pyruvate kinase, glutamate dehydrogenase and alcohol dehydrogenase. PULSAR software (available from http://pulsar.chem.ox.ac.uk/) (Allison et al., 2015) was used to create a calibration curve (Bush et al., 2010) and calculate CCS values for UapA. MOBCAL software (available from http://www.indiana.edu/~nano/software/) was used to calculate the CCS of the UapA crystal structure (PDB: 5I6C) (Shvartsburg and Jarrold, 1996). The CCS of missing residues from the crystal structure was accounted for using the following equation (Hall et al., 2012; Politis et al., 2010):

\[
CCS_{\text{Total}} = 1.14 \times CCS_{\text{PA}} \left( \frac{\text{Mass}_{\text{MS}}}{\text{Mass}_{\text{PDB}}} \right)^{2/3}
\]

Equation 1: CCS_{\text{PA}} refers to the CCS calculated using the proximal approximation via MOBCAL. Mass_{\text{MS}} refers to the mass of the protein analysed by MS. Mass_{\text{PDB}} refers to the mass of protein in the PDB file.

LC-MS

A 10 μL 60 μM UapA sample was subjected to a lipid extraction as described by the Folch method (Folch et al., 1957). The lipid extract was separated by liquid chromatography using an Accela Autosampler (Thermo Scientific) coupled to a LTQ Orbitrap Elite (Thermo Scientific) mass spectrometer. 5 μL lipid extract was separated at a flow rate of 0.5 mL/min on an Acuity C18 BEH column (Waters, 50x2.1mm, 1.7μm) at 55 °C. Mobile phase A = acetonitrile:water (60:40) with 10mM ammonium acetate. Mobile phase B = isopropanol:acetonitrile (90:10) with 10mM ammonium acetate. Lipids were initially separated with 60:40 mobile phase A:B. This mobile phase gradient was linearly changed to 1:99 A:B over 8 minutes and kept constant for 30 seconds. 60:40 mobile phase A:B was then regained in 10 seconds. These conditions were maintained for a further 2.5 minutes. Lipids were analysed by MS in the negative ion mode. Tandem MS was performed to fragment intact lipids in order to identify the fatty acyl chains using their diagnostic ions.

Coarse-grained Molecular Dynamics (CG-MD) Simulations

The CG-MD simulations were performed using the Martini 2.2 force-field (de Jong et al., 2013; Marrink et al., 2007) and GROMACS 4.6 (David Van Der et al., 2005). The crystal structure of UapA dimer (PDB: 5I6C) (Alguel et al., 2016) (residues 66 to 545) was used for the CG-MD simulations. Missing unstructured regions from the crystal structure were added using Modeller (Fiser and Blundell, 1993) prior to the simulations and the V411 mutation in the crystal was mutated to glycine to generate a WT model. We also note that prior to the conversion to the coarse-grained representation, the substrate, xanthine, present in the crystal structure was removed.

A POPC bilayer was self-assembled around the UapA dimer and the snapshot at the end of this simulation was taken. Six different systems were generated with the protein inserted in complex symmetric bilayers that contained the following lipid concentrations: 1) 100% POPC, 2) 100% POPE, 3) 60% POPC-40% POPE, 4) 40% POPC-25% POPE-5% PI, 5) 65% POPC-25% POPE-10% PI, and 6) 70% POPC-25% POPE-5% PI. The exchange of lipids was done as previously described (Koldsø et al., 2014). For the simulations, we have used CG models of POPC and POPE lipids that had 4 CG particles in one of the lipids tails and 5 CG particles in the other tail (with one particle representing the double bond in the chain with 5 particles). Those are the CG-equivalent of the 1-palmitoyl 2-oleoyl-phosphatidylcholine (POPC) and 1-palmitoyl 2-oleoyl-phosphatidylethanolamine (POPE). The PI lipids had a tail with 4 CG particles and a tail with 5 CG particles but with no double bond. CG water particles were added to solvate the simulation systems and the LIPID MAPS database (Fahy et al., 2007). Cardiolipin also co-purified with UapAG411V and the LIPID MAPS database (Fahy et al., 2007). Cardiolipin also co-purified with UapAG411V from the yeast mitochondria (Schneiter et al., 1999).

A. nidulans Growth Conditions, UapA Localisation, Bifluorescence Complementation Assays and Xanthine Transport Assays

An A. nidulans mutant strain (uapA Δ uapC Δ azgA Δ pabaA1 argB2) lacking the genes encoding all major endogenous purine uptake systems was used (Pantazopoulou et al., 2007). This strain will only grow using xanthine or uric acid as the sole nitrogen source when a functional UapA construct is introduced. GFP-tagged UapA constructs (UapA^+, WT), R287A, R478A, R479A, R478A/R479A, R287A/R478A/R479A) were generated and transformed as previously described (Koukaki et al., 2003) into A. nidulans. The GFP...
tag has been shown not to affect UapA localisation, function, or transport kinetics in *A. nidulans* (Pantazopoulou et al., 2007). Successfully transformed strains of *A. nidulans* were selected via arginine auxotrophy complementation (Pantazopoulou et al., 2007).

As previously described (Karachaliou et al., 2013), UapA subcellular localisation was measured by visualising GFP-tagged UapA using epifluorescence inverted microscopy (Zeiss Observer Z1/Axiocam HR R3 camera/Zen lite 2012 software). Radiolabelled \[^{3}H\]-xanthine (22.8 Ci mmol\(^{-1}\), Moravek Biochemicals, CA, USA) uptake was measured using *A. nidulans* germinating conidiospores at 37°C or 25°C, pH 6.8, as previously described (Krypotou and Diallinas, 2014). To measure growth of *A. nidulans* in different nitrogen sources, transformed strains were grown on minimal media supplemented with nitrogen sources (10 mM ammonium tartrate or 0.5 mM uric acid or 0.5 mM xanthine) at 37°C or 25°C, pH 6.8. Dimerisation of mutant UapA was measured via a bimolecular fluorescence complementation assay, as described previously (Martzoukou et al., 2015). In brief, the N-terminal 154 amino acids of YFP or the C-terminal 86 amino acids of YFP were cloned into the pAN510exp or pAN520exp vector at the XbaI site. *uapA* with the necessary mutations was then cloned into the vector and transformed into *A. nidulans*. YFP fluorescence was measured using epifluorescence inverted microscopy (Zeiss Observer Z1/Axiocam HR R3 camera/Zen Lite 2012 software). Relative quantification was carried out using the ICY colocalization studio plugin (pixel-based method) (http://icy.bioimageanalysis.org/) and statistical analysis (Tukey’s Multiple Comparison Test, One-Way ANOVA for n=5 hyphae) of plasma membrane fluorescence intensity of mutants compared to WT UapA+-YFPC/UapA+-YFPN was performed as previously described (Martzoukou et al., 2017).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Bar charts throughout show mean ± standard deviation (n=3, where n represents the number of repeats). For the bimolecular fluorescence complementation analysis using a split YFP system (Figure 5B), statistical analysis (Tukey’s Multiple Comparison Test, One-Way ANOVA for n=5 hyphae) of plasma membrane fluorescence intensity of mutants compared to WT UapA+-YFPC/UapA+-YFPN was performed as previously described (Martzoukou et al., 2017). No additional statistical tests were undertaken.