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How to cite this publication
Please cite the final published version:

*Biochemistry* 2020, 59, 45, 4407–4420

Publication metadata

**Title:** Mechanistic Insight into Lipid Binding to Yeast Niemann Pick Type C2 Protein

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**Journal:** *Biochemistry*

**DOI/Link:** [10.1021/acs.biochem.0c00574](http://10.1021/acs.biochem.0c00574)

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Mechanistic Insight into Lipid Binding to Yeast Niemann Pick type C2 protein

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Abstract

Niemann Pick Type C2 (NPC2) is a small sterol-binding protein in the lumen of late endosomes and lysosomes. We showed recently that the yeast homolog of NPC2, together with its binding partner NCR1 mediates integration of ergosterol, the main sterol in yeast, into the vacuolar membrane. Here, we study the binding specificity and the molecular details of lipid binding to yeast NPC2. We find that NPC2 binds fluorescence- and spin-labeled analogues of phosphatidylcholine (PC), phosphatidylyserine (PS), phosphatidylinositol (PI) and sphingomyelin (SM). Spectroscopic experiments show that NPC2 binds lipid monomers in solution but can also extract lipid analogues from membranes. We further identify ergosterol, PC and PI as endogenous NPC2 ligands. Using molecular dynamics simulations, we show that NPC2’s binding pocket adapt to ligand shape and closes around bound ergosterol. Hydrophobic interactions stabilize the binding of ergosterol, but binding of phospholipids is additionally stabilized by electrostatic interactions at the mouth of the binding site. Our work identifies key residues that are important in stabilizing phospholipid binding to yeast NPC2, thereby rationalizing future mutagenesis studies. Our results suggest that yeast NPC2 functions as general ‘lipid solubilizer’ and binds a variety of amphiphilic lipid ligands, possibly to prevent lipid micelle formation inside the vacuole.
Introduction

Uptake of cholesterol derived from ingestion of low density lipoprotein (LDL) in mammalian cells critically depends on two proteins; Niemann Pick type C1 (NPC1), a large transmembrane protein residing in the limiting membrane of late endosomes and lysosomes (LE/LYSs) and Niemann Pick type C2 (NPC2), a small soluble protein in the lumen of LE/LYSs.\textsuperscript{1,2} NPC2 adopts an Ig-like beta sandwich fold, forming a single binding pocket for sterols.\textsuperscript{3,4} Based on structural and mutagenesis studies, a model has been put forward, in which NPC2 picks up LDL-derived cholesterol and transfers it to NPC1 for membrane integration.\textsuperscript{5–7} The yeast homologs of both NPC proteins, NCR1 and NPC2, show high structural and functional resemblance to their mammalian counterparts. The structures of NCR1 and NPC2 have been solved recently by X-ray diffraction and cryo-electron microscopy.\textsuperscript{8} Both the NTD of NCR1 and yeast NPC2 bind radioactive cholesterol as well as dehydroergosterol (DHE), a fluorescent sterol differing from yeast’s native sterol ergosterol only by having one additional double bond in the steroid ring system. Förster resonance energy transfer from aromatic residues of either NPC2 or the NTD of NCR1 to DHE could be used to demonstrate sterol transfer between both proteins.\textsuperscript{8} We showed recently that this transport system is directly responsible for the integration of ergosterol into the vacuole.\textsuperscript{8} Like the lysosome in mammalian cells, the yeast vacuole is the key organelle for digestion of lipids and proteins and for nutrient sensing. Using a live-cell imaging assay based on DHE, we found that NCR1 and NPC2 are required to deliver sterols to the vacuolar membrane, especially when yeast cells are kept under starvation conditions.\textsuperscript{8}

Bovine NPC2 has been shown to bind a variety of sterols besides cholesterol, including cholesterol sulfate and, though with lower affinity compared to cholesterol, certain oxysterols and, even weaker, the hydrophobic amine U18666A.\textsuperscript{3,9–11} Yeast NPC2 has also been shown to bind cholesterol, ergosterol, DHE and U18666A, but also edelfosine, a phosphatidylcholine-like lysophospholipid, suggesting that its binding spectrum is rather broad.\textsuperscript{8} While the overall structure of mammalian and yeast NPC2 are similar, the binding site for yeast NPC2 is sig-
nificantly larger and more open. This difference suggests that yeast NPC2 could eventually bind other ligands than mammalian NPC2. Supporting that notion is a recent study by Storch and colleagues, showing that mammalian NPC2 contains a hydrophobic knob located in a loop close to the entrance of the sterol binding site, which is required for NPC2’s interaction with lysobisphosphatidic acid (LBPA). LBPA is a special lipid found in LE/LYSs of mammalian cells, where its abundance is linked to cholesterol availability for efflux. LBPA is not found in yeast, and yeast NPC2 does not appear to have a hydrophobic knob. To better understand ligand specificity and binding modes of yeast NPC2, we carry out a combined spectroscopic and computational analysis of this protein in the presence of various lipid analogues. We find that yeast NPC2 binds fluorescence- and spin-labeled phospholipids with a preference for the charged phosphatidylserine (PS) compared to the zwitterionic phosphatidylcholine (PC) and sphingomyelin (SM). Extraction of endogenous lipids from purified NPC2 identified ergosterol, PC and phosphatidylinositol (PI) as native ligands. Molecular dynamics (MD) simulations identify key residues stabilizing the binding of such diverse lipid ligands and further reveal that NPC2’s binding pocket can flexibly adapt around sterols and phospholipids. Implications of these results for the physiological function of yeast NPC2 inside the vacuole and of mammalian NPC2 in the endo-lysosomes are discussed.

Materials and Methods

Fluorescent lipids

18:1-12:0 NBD-PC, 1-oleoyl-2-[12-|(7-nitro-2-1,3-benzoxadiazol-4-yl)amino|dodecanoyl]-sn-glycero-3-phosphocholine (NBD-C12-PC), 16:0-06:0 NDB PC, 1-palmitoyl-2-6-[|(7-nitro-2-1,3-benzoxadiazol-4-yl)amino|hexanoyl-sn-glycero-3-phosphocholine (NBD-C6-PC), 18:1 NBD PE, 1-oleoyl-2-[12-|(7-nitro-2-1,3-benzoxadiazol-4-yl)amino|dodecanoyl]-sn-glycero-3-phosphoethanolamine (NBD-C12-PE), 18:1-12:0 NBD PS, 1-oleoyl-2-12-[|(7-nitro-2-1,3-benzoxadiazol-4-yl)amino|dodecanoyl-sn-glycero-3-phosphoserine (ammonium salt) (NBD-C12-PS), C12-NBD SM, N-[12-|(7-nitro-
2-1,3-benzoxadiazol-4-yl)amino@dodecanoyl]-sphingosine-1-phosphocholine (NBD-C12-SM) and 
NBD-C6-Cl\textsuperscript{14} were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). NBD-
tagged ether lipids were synthesized as previously described.\textsuperscript{14} 16:0-06:0 NDB PI, 1-palmitoyl-
2-6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl-sn-glycero-3-phosphatidylinositol (NBD-
C6-PI) was kindly provided by Prof. Bütkofer (University of Bern, Switzerland).\textsuperscript{15} DHE 
was purchased from SIGMA Aldrich.

**Preparation of large unilamellar vesicles (LUVs)**

For the lifetime-based extraction assay, the proper volume of lipids (POPC and C12-NBD-PC 
in a ratio of 9:1) was dissolved in chloroform followed by evaporation in a rotary evaporator 
for 20 min, leaving a thin lipid film, which was re-solubilized in 1 mL M\textsuperscript{16} buffer by vortexing, 
giving a 1 mM multilamellar vesicle suspension. This vesicle suspension went through 5x 
freeze/thaw cycles for formation of LUVs, followed by extrusion 10 times through a 100 nm 
por membrane, yielding LUVs of approx. 100 nm in diameter. Unilamellarity was 
checked with a dithionite assay (see below). For the dithionite assay, the same preparation 
protocol was used, except that the lipid composition of LUVs consisted of 80% POPC, 19.5% 
cholesterol and 0.5% C12-NBD-PC.

**Protein purification, lipid extraction and thin layer chromatography**\textsuperscript{17}

NPC2 containing a C-terminal purification tag was purified from the *S. cerevisiae* expression 
strain DSY-5 as previously described.\textsuperscript{8} In brief, cells were resuspended in L\textsuperscript{18} buffer (600 
mM NaCl, 100 mM Tris pH 7.5), then lysed using a bead beater. After ultracentrifugation, 
filtered supernatant was applied to a nickel column (HisTrap\textsuperscript{TM}) and washed with 20 column 
volumes (\textsuperscript{20}) W70 buffer (500 mM NaCl, 50 mM Tris pH 7.5, 10 % glycerol, 70 mM 
imidazole, 0.017 % N-dodecyl-beta-D-maltoside (DDM)). When purifying NPC2 for TLC, 
the W70 did not contain any DDM. The column was then washed with 9 CV G20 buffer 
(200 mM NaCl, 20 mM Tris pH 7.5, 20 mM imidazole). The protein was eluted from the
column in 5 ml of G20 buffer with 175 units of bovine thrombin and 2000 units Endo-H circulating over the column overnight. Elution of protein was finalized with a run with 10 mL G20 buffer. All the eluant was collected and concentrated, followed by size exclusion chromatography (Superdex 75 10/300 GL) using SEC buffer (200 mM NaCl, 20 mM Tris pH 7.5). For extraction of endogenous ligand, purified NPC2 (250 µL @ 1.2 mg/mL) was mixed with CHCl$_3$/MeOH/H$_2$O 65:25:4 (v/v/v) at a 1:3 ratio in a glass vial. The chloroform phase was transferred to a new vial and evaporated to a volume of 10 µL with N$_2$ gas. Lipid standard samples (Soy phosphatidic acid (PA), Soy PC, Soy PE, Soy phosphatidylglycerol (PG), Soy PI, Soy PS, Ergosterol, Yeast Polar Lipid Extract) were rapidly deposited on TLC plates (ALUGRAM TLC sheets, 0.2 mm silica gel, MACHERY-NAGEL) as 1 cm parallel streaks (2 µL @ 10 mg/mL in chloroform). All of the NPC2 extracted lipid samples were deposited next to the standards. TLC plates were placed in a glass beaker, lined with filter paper and equilibrated for at least 1 h with chloroform/ethanol/water/tri-ethylamine 30:35:7:35 (v/v/v/v) prior to the experiment, and covered with a lid. After 1 hour migration, the plates were dried in the fume hood and sprayed with developing solution (50 % ethanol, 3.2 % H$_2$SO$_4$, 0.5 % MnCl$_2$) followed by charring on a hot plate at 120 degrees C for 30 min.

**Fluorescence spectroscopy of ligand binding**

**Binding at equilibrium**

Aliquots of fluorescent lipid analogues dissolved in chloroform were deposited into a glass vial. Chloroform was evaporated under a stream of nitrogen. The analogues were resuspended in MES buffer (500 mM NaCl, 50 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.5) giving a 100-µM stock solution. An aliquot of this lipid suspension was added to a cuvette with an optical path length of 1 cm at a final concentration of 1 µM. Purified NPC2 was added from a stock solution in MES buffer, and the sample was incubated for 15-20 min at room temperature to ensure that a binding equilibrium was reached. Emission scans were carried out for excitation at $\lambda_{ex} = 460$ nm in the range of 480-600 nm using an Aminco
Bowman spectrometer series 2 (Urbana, IL, USA).

**Stopped flow measurements**

Measurements were recorded with $\lambda_{ex} = 460$ nm and $\lambda_{em} = 540$ nm using a stopped-flow device (RX 1000 Rapid Kinetics, Applied Photophysics, Surrey, UK) connected to an Aminco Bowman spectrometer series 2. A 20 µM solution of NBD tagged ligands was loaded into one syringe, while the other contained a 4 µM NPC2 solution, yielding a 1:5 protein/lipid ratio after mixing. Fluorescence was measured for 10 min with a time resolution of 1 s at room temperature, however, for faster kinetics, e.g., for DHE and C6-NBD-PC, the resolution was set to 10 ms.

**Fluorescence lifetime measurements**

For measurement of binding of NPC2 to lipid analogues in suspension, a 10 µM solution of NBD tagged lipid was added into a 1x1 cm cuvette, either with or without 2 µM NPC2. Fluorescence lifetimes of NBD-lipids in the solution were measured by time-correlated single photon counting using a FluoTime 200 Time-resolved Spectrometer (Picoquant, Berlin, Germany). A 467-nm laser was used as excitation source, and emission was collected at 540 nm. Data were acquired up to 20000 counts (unless stated otherwise), which was based on the maximum of the fluorescence lifetime decay curve. For measurement of NPC2-mediated lipid extraction from liposomes, LUVs made of POPC with C12-NBD-PC in a 9:1 ratio (see above) were added to a 1x1 cm cuvette to a total lipid analogue concentration of 10 µM in MES buffer (pH 5.5). Fluorescence lifetime of C12-NBD-PC was measured at 540 nm upon excitation at 460 nm, either in the absence or presence of 2 µM NPC2, and seven measurements were averaged for each condition.
Dithionite quenching experiments

Sodium dithionite is an irreversible membrane-impermeable quencher of NBD fluorescence, which rapidly reacts with the fluorophore to form a non-fluorescent product, thereby allowing for selective assessment of leaflet-specific processes in model and cell membranes.\textsuperscript{16,17} Dithionite quenching measurements were done on LUVs using an Aminco Bowman spectrometer series 2 (Rochester, USA). A 1 mL solution of 1M sodium dithionite was prepared in HBS buffer (pH 10). For each measurement, 50 µL of 0.2 mM LUVs in the absence or presence of 20 µM NPC2 were diluted in HEPES buffer, a final volume of 1.5 mL. Fluorescence time traces of C12-NBD-PC were measured at 540 nm for an excitation of 470 nm. First, a baseline intensity was measured before dithionite was injected with a Hamilton syringe to a final concentration of 25 mM. Fluorescence intensity was recorded under constant stirring for 400 sec. Triton X-100 (1% v/v final amount) was added after 370 sec recording to disrupt LUVs and thereby give dithionite access to remaining NBD-lipids on the inner membrane leaflet. Quenching kinetics was fitted with a bi-exponential decay model.

Measurement of ligand binding by ESR spectroscopy

ESR spectra of a 100 µM solution of spin-labeled lipids in the absence or presence of 10 µM NPC2 were measured at room temperature, using a Bruker EMX spectrometer (Bruker, Karlsruhe, Germany). The measuring parameters were set to a modulation amplitude of 1 G, a power of 20 mW, scan width 100 G and four times accumulation. Samples were measured either in HEPES buffer (150 mM NaCl, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5) or in citrate buffer (11.5 mM Na-citrate, 3.5 mM citric acid, pH 5.5).
Quantification of binding from spectroscopic measurements

Fluorescence measurements were first normalized according to:

\[ F = \frac{F_{meas} - F_0}{F_{max} - F_0} \]  

(1)

Here, \( F_{meas} \) is the measured fluorescence output, \( F_0 \) is the probe fluorescence in the absence of NPC2, and \( F_{max} \) is the maximal fluorescence signal of a given measurement. Binding isotherms were fitted to a one-site binding model, as described in Ref. 8, with the modification that the lipid was considered as a receptor of fixed concentration of \( R_T = 1 \, \mu\text{M} \), while the protein was considered as the ligand with varying concentration \( L_T \). This leads to the following expression for fractional saturation of the lipid receptor with protein ligand

\[ \frac{R_L}{R_T} = \frac{(L_T + R_T + K_D) + \sqrt{(L_T + R_T + K_D)^2 - 4L_T R_T}}{2R_T} \]  

(2)

where \( R_L \) is the concentration of receptor-ligand complex. For binding experiments of analogues below their respective CMC, a simple hyperbolic binding model was additionally used:

\[ \frac{R_L}{R_T} = \frac{L}{L + K_D} \]  

(3)

Here, \( L \) is the concentration of free ligand (NPC2), which is assumed to equal the total ligand concentration. Stopped-flow binding kinetics were fitted to either a bi-exponential or a stretched exponential rise-to-maximum model, as indicated in the figures.

Fluorescence lifetime decays were fitted with a bi-exponential decay model.

\[ F(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} \]  

(4)

Here, \( a_1 \) and \( a_2 \) are the fractional amplitudes, while \( \tau_1 \) and \( \tau_2 \) are time constants for both kinetic components. From those values, fractional contributions are calculated for each decay
component, \( i = 1, 2 \), according to:

\[
f_i = \frac{a_i \tau_i}{a_1 \tau_1 + a_2 \tau_2}
\]  

(5)

From that, the average time constant for the bi-exponential fluorescence decay can be calculated as:

\[
\langle \tau \rangle = f_1 \tau_1 + f_2 \tau_2
\]  

(6)

**Computational Details**

**Preparation of NPC2 Protein and Ligands**

The crystal structure of the NPC2 protein was acquired from the Protein Data Bank (PDB ID: 6R4N, X-ray crystal structure resolution 2.8 Å)\(^8\) and imported into the Maestro module, which is available in the Schrödinger Suite.\(^18\) Chain A from the crystal structure was extracted and prepared as detailed in the following. The sulfate ions present in the X-ray structure and an N-acetylglucosamine were removed, and the protein was prepared using the Protein Preparation Wizard, which includes added missing hydrogen atoms and assigning bond orders. Protonation states (at pH=6) were determined by the PROPKA\(^{19,20}\) tool included in the Protein Preparation Wizard. Finally, the structure was minimized using the OPLS3e force field, keeping the positions of the heavy atoms fixed. Three lipids (POPC, POPI and POPS) were constructed and processed by the LigPrep\(^21\) tool, which uses the OPLS3e force field to minimize the energies of the structures.

**Initial pose generation**

Initial positions of POPC, POPI and POPS at the binding site were determined by docking the ligands to the prepared protein using the Glide program.\(^22\) A docking grid was generated around the active site defined from the centroid of the ergosterol and expanded so that it covered the entire cavity of the protein. The grid was extended in diameter from the initial
ergosterol molecule to cover the entire binding cavity. The docking was performed in the XP-docking (eXtra Precision) mode. Multiple binding poses were found using this method. A single binding pose for each lipid was selected for the MD simulations based on the criteria that the tails were positioned inside the cavity. For ergosterol, the binding pose in the crystal structure was used.

Molecular Dynamics Simulations

All MD simulations were performed using the Amber 16 software. The tleap tool in Amber was used to create topology and coordinate files using the Amber ff14SB (protein), TIP3P (water) and GAFF2 (lipids) force fields. Charges for the lipids were assigned based on electrostatic potential (ESP) fitting with Antechamber, which also assigned atom types. The ESP used in the fit was obtained with Gaussian09 based on HF/6-31G* optimized structures. This ESP was then used in a RESP fitting procedure, performed by Antechamber. TIP3P water was used to solvate the systems, with a buffering distance of 12 Å. Salt ions were added to neutralize the system and to obtain a salt-ion concentration of 150 mM.

Energy minimizations were performed with constraints on heavy atoms and a maximum of 1000 cycles, where the first 500 iterations were using the steepest descent algorithm, and the rest used the conjugate gradient algorithm. The system was then heated to 300 K in the span of 50 ps using the Langevin thermostat, followed by 50 ps of density equilibration using the Berendsen barostat. The systems were then set to equilibrate (with production settings) for 500 ps at constant pressure and temperature of 300 K. Initial velocities were generated from random seeds based on a Maxwell-Boltzmann distribution, and bonds involving hydrogen were constrained using the SHAKE algorithm. All MD simulations were run using a time step of 2 fs. Following this initial short equilibration step, longer simulations of 300 ns were carried out with production settings. Only the last 100 ns were used for analysis. Coordinates were saved every 10 ps for analysis, which left 10000 snapshots for analysis.
Molecular Mechanics Poisson-Boltzmann Surface Area Calculations

The MMPBSA.py script\textsuperscript{33} was used to estimate binding affinities for all protein-ligand complexes based on the Molecular Mechanics Poisson-Boltzmann Surface Area (MM-PBSA) method.\textsuperscript{34} The calculations were performed based on every 6th snapshot extracted from the simulations. Thus, from the subsequent production phases, 1667 snapshots were generated for MM-PBSA calculations. Entropic contributions were not included.

Results and Discussion

Spectroscopic studies of ligand binding

Fluorescent analogs of phospholipids and sterols bearing a nitrobenzoxadiazole (NBD) group are widely used in ligand binding studies.\textsuperscript{35} NBD-lipids show very low fluorescence in aqueous environments due to efficient self-quenching in micelles and molecular aggregates.\textsuperscript{36,37} In the case of cholesterol analogs, proper localization of the NBD group on the sterol moiety is important to minimize interference with the binding process.\textsuperscript{36,38} Given the orientation of ergosterol in the binding pocket of yeast NPC2,\textsuperscript{8} we have chosen NBD-cholesterol with the fluorescent moiety linked via a short acyl chain at the 3'-hydroxy group of cholesterol (NBD-C6-CHOL, see Fig. S1 for its structure). NBD-C6-CHOL will form micelles in aqueous solution, in which its fluorescence is self-quenched. We found that the fluorescence of NBD-C6-CHOL increases upon binding to NPC2, which can be employed to determine its binding affinity from a binding isotherm (Fig. 1A and B). Relief of self-quenching upon binding to NPC2 is likely a consequence of solubilization of sterol micelles by the protein, which efficiently shifts the equilibrium of NBD-C6-CHOL from the micelle to the NPC2-bound monomer form. Binding of NBD-C6-CHOL to NPC2 was further seen as an increase of the fluorescence lifetime of NBD-C6-CHOL in the presence of the protein (Fig. 1C and Supplemental data). The binding kinetics of NBD-C6-CHOL was determined using a fluo-
nescence spectrometer equipped with a stopped-flow device and was found to be rather slow and of sigmoid shape (Fig. 1D). We compared the kinetics of binding of NBD-C6-CHOL to NPC2 to that of the fluorescence ergosterol analog DHE, which, as we showed recently, binds to yeast NPC2 with sub-micromolar affinity. DHE differs from the native yeast sterol ergosterol only in having one additional double bond (Fig. S1). Its binding to proteins can be measured by resonance energy transfer from aromatic residues, but DHE also shows a slight increase in quantum yield upon binding, which can be explored to study its interaction with NPC2 (Fig. S2). Using this readout, we find that DHE binds to yeast NPC2 more rapidly compared to NBD-C6-CHOL (compare Fig. 1D and S2). Also, the dissociation constant from NPC2 is smaller for DHE ($K_D=0.121 \text{ \mu M}$) than for NBD-C6-CHOL ($K_D=0.375 \text{ \mu M}$). Thus, while the NBD-moiety affects the kinetics and strength of ligand binding to NPC2, the results with NBD-C6-CHOL also show that the NBD moiety provides a sensitive readout for binding studies, which can be employed to determine binding of other lipids, i.e., NBD-labeled phospholipids.

To identify additional ligands of yeast NPC2, we used phospholipid analogs bearing an NBD-group at the C12-position of the sn2-acyl chain (Fig. S1). Fluorescence emission and lifetimes of both C12-NBD-PC and C12-NBD-PS increased strongly upon the addition of NPC2 (Fig. 2 and Supplemental data). This demonstrates that self-quenching of the analogues is relieved upon binding to NPC2, and it suggests that the NBD-moiety attached to these phospholipid analogues gets accommodated in the hydrophobic binding pocket of the protein.

When plotting the fluorescence maxima of C12-NBD-PC and -PS shown in Fig. 2A and C as a function of the NPC2 concentration, the half-maximal fluorescence was reached for much lower protein concentrations for the analog of PS compared to that of PC (Fig. 3). From the averaged and normalized fluorescence increase, we estimated a dissociation constant of $K_D=0.088 \text{ \mu M}$ and $K_D=0.972 \text{ \mu M}$ for C12-NBD-PS and -PC, respectively. The binding kinetics measured in a stopped flow apparatus was comparable for both phos-
Figure 1: NBD-tagged cholesterol (NBD-C6-CHOL) binds to yeast NPC2. A, emission spectrum of NBD-C6-CHOL for increasing concentrations of NPC2. B, normalized fluorescence from (A) plotted as a function of NPC2 concentration. Data was fitted to a one-side saturation binding model as described in Materials and Methods. C, fluorescence lifetime of NBD-C6-CHOL excited at 461 nm and measured at 540 nm in the absence (blue) or presence of NPC2 (red). D, kinetics of fluorescence increase of NBD-C6-CHOL upon binding to NPC2 measured in a stopped flow apparatus at excitation and emission of 460 and 540 nm, respectively. Dots are data (mean of 3 measurements ± standard deviation. The red line is a fit to a compressed exponential function.
Figure 2: Fluorescence changes of NBD-tagged PC and PS in the presence of NPC2. A, C, Fluorescence emission spectra of 1 µM NBD-C12-PC (A) and C12-NBD-PS (C) were recorded after addition of NPC2 (concentrations, see inset) and a 15 min incubation at an excitation wavelength of 460 nm. B, D, Lifetime measurements of 10 µM of C12-NBD-PC (B) and C12-NBD-PS (D) in the presence (orange) or absence (blue) of 2 µM NPC2. Excitation and emission were set to 460 and 540 nm, respectively.
pholipid analogues and rather slow, as completion of binding took about 5 min, when a stable plateau value of fluorescence was reached (Fig. 3C and D). Given the different estimates for the dissociation constants despite comparable binding kinetics, one can conclude that the free energy difference between free and bound ligand is much higher for C12-NBD-PS than for C12-NBD-PC, while the activation energy for complex formation of both phospholipid analogs with NPC2 is comparable.

To assess the impact of the length of the fatty acyl chain on which the NBD group is located, we repeated the binding studies for a PC analogue with NBD moiety on a 6-carbon acyl chain in the sn2 position (Fig. S3). We found a higher binding affinity ($K_D=0.492 \mu M$ and $K_D=0.972 \mu M$ for C6- and C12-NBD-PC, respectively) and much faster binding kinetics for short-chain PC compared to C12-NBD-PC. These results could indicate that NPC2 preferentially binds analogs with shorter acyl chain length. However, the difference in binding kinetics and dissociation constants could also be a consequence of the different critical micellar concentrations (CMCs) and aggregate structures of short- versus long-chain phospholipid analogs. While C6-NBD-PC likely forms spherical or prolate-shaped micelles, the long chain analogue C12-NBD-PC might form bilayer structures, as it has almost cylindrical shape, for which the optimal packing is in bilayer assemblies. We measured the CMC of C6-NBD-PC previously by a self-quenching approach and found a value of 0.116 $\mu M$. For C12-NBD-PC the CMC (or equivalently critical aggregation concentration) was too low to be resolvable by our approach, which was sensitive down to 10 nM. As the CMC of phospholipid analogues, in which only one acyl chain length is varied, drops by about a factor of ten for every two methyl groups, the CMC of C12-NBD-PC must be lower than 1 nM. Thus, for both, short and long chain phospholipid analogs, the CMC is lower than the measured $K_D$-value in binding to NPC2. This raises the question, whether NPC2 can directly pick up phospholipid monomers in solution or whether it requires the interfacial environment of the phospholipid micelle/bilayer to extract lipid monomers for binding. In an attempt to answer this question, we have measured binding of C6-NBD-PC to NPC2 when the lipid analogue concentration
Figure 3: Comparison of binding of NBD-tagged PC and PS to NPC2.

Fluorescence emission maxima of C12-NBD-PC (A) or C12-NBD-PS (B) at 524 nm were normalized and plotted as a function of NPC2 concentration. Data was fitted to a one-side saturation binding model as described in Materials and Methods. The kinetics of binding was determined using a stopped flow device. Ten µM of C12-NBD-PC (C) or C12-NBD-PS (D) were mixed with 2 µM NPC2. The increase of fluorescence (excitation 460 nm, 540 nm) upon mixing was measured with a time resolution of 1 sec. Data was fitted to a bi-exponential model. Data represent mean +/- standard deviation of two (A and B) or 5 measurements (C and D). All measurements were done in MES buffer (pH 5.5).
was below its CMC (Fig. S4). We found that the fluorescence of monomeric C6-NBD-PC increases upon binding to NPC2, likely because the apolar environment of the protein binding pose results in an increased quantum yield of the NBD-group. By fitting the binding model of Eq. 2 to this data, we obtained a dissociation constant for binding of monomers of C6-NBD-PC to NPC2 of $K_D=3.6$ nM (Fig. S4). This is more than 100-fold smaller than the measured dissociation constant above the CMC, but, since the mechanism by which binding increases NBD-fluorescence likely differs below and above the CMC, a direct comparison of these $K_D$ values should be avoided. Also, a simpler hyperbolic binding model gave a better fit, suggesting that fluorescence responses of NBD-tagged lipids upon binding to NPC2 below and above their CMC cannot be compared directly. The experiment nevertheless shows, that NPC2 can indeed acquire phospholipid monomers directly in the solution. In the presence of lipid micelles, binding of monomers to NPC2 will likely shift the equilibrium between micelles and monomers thereby replenishing further monomers for binding. As the residence time of phospholipid molecules in a micelle is approximately inversely proportional to the CMC, the different binding kinetics of short- versus long-chain PC analogues to NPC2 will also be affected by the lipid aggregation state. Furthermore, replenishment of phospholipid analogues from the inner leaflet of bilayer assemblies or even inner membranes in case of multilamellar liposome-like structures could significantly delay binding of long-chain NBD-tagged lipids to NPC2 compared to their short-chain counterparts which organize in micelles.

To directly test these notions, we prepared large unilamellar vesicles containing C12-NBD-PC and assessed the impact of NPC2 on accessibility of the lipid probe with the quencher sodium dithionite. Adding dithionite in the absence of NPC2 resulted in a biphasic fluorescence quenching kinetics, in which the first phase is due to the quenching reaction in the outer membrane leaflet, and the second phase due to flip-flop of the lipid and/or slow permeation of the quencher (Fig. S5). In the presence of NPC2, both kinetic phases were accelerated, demonstrating that NPC2 does interact with the lipid bilayer. That NPC2
accelerates the quenching reaction, the first phase, suggests that the protein extracts some C12-NBD-PC, which thereby easier can react with dithionite.\textsuperscript{17,42} That NPC2 enhances the second quenching phase shows that it additionally affects lipid packing and/or flip-flop in the bilayer.\textsuperscript{42,43} More evidence for extraction of C12-NBD-PC from a membrane by NPC2 comes from our observation that the fluorescence lifetime of C12-NBD-PC is increased upon addition of the protein to a solution of LUVs (Fig. S5). Together, we conclude that NPC2 can directly bind lipid monomers in the solution but can also extract lipid analogs from membranes and will likely interact with micelles and other lipid assemblies. The latter is further supported by the observed binding of an analogue of phosphatidylethanolamine (PE), a lipid preferring inverted-hexagonal phases, to NPC2 (not shown, but see fluorescence lifetime information in Supporting material). The chain-length dependent equilibrium between phospholipid monomers and phospholipid assemblies will have a non-negligible impact on the measured binding affinity and kinetics of phospholipid analogues to NPC2.

We also assessed the impact of head group linkage of phosphocholine containing lipids on binding to NPC2. Here, we found that NPC2 also binds the sphingomyelin analogue C12-NBD-SM with comparable characteristics compared to C12-NBD-PC, though with even slower kinetics (Fig. S6). As PC and SM share the same head group but differ in their backbone structure, it is unlikely that the head group linkage’s particular nature plays a decisive role in the binding process. Similarly, we found that ether-linked acyl chains in NBD-PC did not interfere with binding to NPC2 (Fig. S7).

Yeast vacuoles harbor a reasonable amount of phosphatidylinositol (PI) lipids,\textsuperscript{44} and given that we observed strong binding to the negatively charged PS analogues compared to analogues of PC, we speculated whether NPC2 eventually also binds the negatively charged PI. To test this notion, we measured binding of NPC2 to a C6-NBD-tagged phosphatidylinositol, C6-NBD-PI. Based on the large inositol head group (Fig. S1), we surmised, that C6-
NBD-PI has a higher CMC than C6-NBD-PC. By measuring the CMC using the procedure described previously,\textsuperscript{40} we found a value of 2.3 $\mu$M for C6-NBD-PI, which is about twice the value of C6-NBD-PC (not shown).\textsuperscript{40} Since the CMC of C6-NBD-PI is thereby higher than the maximal total lipid concentration in all our binding assays (i.e. $>1\mu$M), an analogue concentration below its CMC (i.e. 1 $\mu$M) was chosen in the following binding experiments. We observed strong binding of C6-NBD-PI to NPC2 with nanomolar affinity, similar to binding of C6-NBD-PC below the CMC (compare Fig. S4 and S8). The binding curve was again better described by a simplified hyperbolic model (Eq. 3) than by the full binding model (Eq. 2), suggesting that the fluorescence response of monomeric NBD-lipids somehow differs from NBD-lipids above their CMC (Fig. S8B, orange and green curve). Together, these experiments demonstrate that NPC2 binds a variety of fluorescent phospholipid analogues. A summary of all lifetime measurements of analogs in suspension in response to NPC2 binding is given in Fig. S9.

To validate the results obtained with fluorescent phospholipids, we performed binding experiments using differentially labeled lipids, i.e., spin-labeled phospholipids in conjunction with ESR spectroscopy. For that, we focused on the binding of NPC2 to an analogue of PS bearing a nitroxid group at a short sn2 fatty acyl chain (SL-PS, see Fig. S1). The ESR spectrum of SL-PS in buffer at 100 $\mu$M, which is well above its CMC of 13.2 $\mu$M,\textsuperscript{40} consists of two superimposed components; (i) three narrow lines arising from the freely tumbling monomers and (ii) a broad component caused by the spin-spin interaction of analogues organized in micelles (Fig. 4, blue spectra). Thus, ESR spectroscopy allows for following both the fraction of lipid in micelles and free in solution independently. Any change of the equilibrium between micelles and monomers, e.g., due to binding of the analog to a protein can be observed by spectral changes.\textsuperscript{45} After addition of NPC2 to an aqueous dispersion of SL-PS, the shape of the ESR spectrum is changed, i.e., the signal intensity of the narrow peaks is increased while the intensity of the broad component is decreased. This indicates
some solubilization of analogue micelles probably caused by binding of SL-PS monomers to NPC2. We measured binding of SL-PS to NPC2 at acidic pH, as found in the vacuole, but also at neutral pH and did not find any difference (Fig. 4). Together, these results demonstrate that binding of phospholipid analogs to NPC2 is not a result of a particular label moiety. Our spectroscopic experiments further show that NPC2 binds not only sterols but also a variety of fluorescent phospholipid analogs, either as monomers or in lipid assemblies.

Figure 4: Binding of spin-labeled PS to NPC2. ESR spectra of 100 µM SL-PS in buffer were measured without (blue spectra) and with 10 µM NPC2 (orange spectra). Measurements were carried out either in HEPES buffer (pH 7.5, A) or in citrate buffer (pH 5.5, B).

**Binding of endogenous lipids to yeast NPC2**

To assess the physiological relevance of these findings, we next determined whether yeast NPC2 also binds endogenous phospholipids *in vivo*. For that, we isolated NPC2 from yeast and extracted lipids bound to the purified protein. The identity of the lipids was determined by Thin Layer Chromatography (TLC) by comparing extracts to known standards. We found that NPC2 binds, beside ergosterol, PC and PI, together with traces of other lipid species (Fig. 5). These results confirm the spectroscopic measurements with fluorescent analogs of PC and PI and fluorescent sterols. PS could not be identified as an endogenous
ligand, which - at first glance - is in contradiction to our spectroscopic studies. However, PS is known to be a minor component of the vacuolar membrane under normal conditions (i.e., it amounts to only 4.4 percent of total phospholipid), while both, PC and PI, are very abundant with 46.5 and 18.3 percent, respectively. Thus, given the low abundance of PS one can expect, despite a significant affinity of PS for NPC2, that only a few protein-PS complexes are formed in the vacuole at any given time, which are hard to detect. Our results show that NPC2 binds phospholipids also \textit{in vivo} supporting a model of NPC2 mediated solubilization of phospholipids in the vacuole.

\textbf{Computational investigation of lipid binding}

To identify structural and energetic determinants of NPC2’s multi-ligand specificity, we used molecular simulations of NPC2 ligand complexes. A series of five MD simulations were performed to investigate how the NPC2 protein binds different lipids: a simulation of the protein with no lipid bound (apo) and simulations of the protein in complex with ergosterol, POPI, POPC, or POPS. Figure 6 contains a comparison of snapshots based on the initial conformation from the simulations to averaged structures of the last part of the simulations. Significant changes to the protein conformation are observed in all of the five simulations. NPC2 has a binding cavity comprised of apolar residues while the surface of the protein has a high number of charged residues. The binding cavity is large compared to the size of ergosterol, which allows water to enter the cavity in the ergosterol and lipid-free simulation. Interestingly, however, this binding pocket closes rapidly in the early stages of the unbound protein simulation and thereby repels water from the apolar binding pocket. This strongly suggests that the binding pocket is very flexible, which likely explains the ability of the protein to bind a wide range of lipids. Note, that the binding pocket in the crystal structure of the lipid-free NPC2 could contain a different lipid, whose identity could not be resolved. In fact, only ergosterol could be resolved but several NPC2 monomers had additional density that could indicate that other lipids were also bound. We couldn’t model such density, likely
Figure 5: Identification of endogenous lipids bound to yeast NPC2. Lipids were extracted from purified NPC2 and analyzed by TLC compared to various standards including phosphatidylglycerol (PG), PC, ergosterol (ERG), phosphatidic acid (PA), PI and phosphatidylethanolamine (PE). In addition, a yeast total lipid extract (Yeast Polar) was used. Ergosterol (left side, upper arrow), PC (left side, lower arrow) and PI (right side, arrow) were identified as ligands of NPC2.
because NPC2 binds lipid ligands with high variability\textsuperscript{8}.

The same dynamic adjustment of the binding pocket as in the apo-state is observed in the simulation of NPC2 in complex with ergosterol. Since ergosterol is the native ligand of NPC2, we did not expect that the conformation of the protein would change much when simulating the protein in complex with this sterol. However, as seen in Figure 6, the sterol moves deeply into the binding pocket, and the protein closes around it to provide a stable, closed conformation indicating tight binding. This movement happens within the first 50 ns of the simulation (see Supplemental video). In the average structure, based on snapshots from the last part of the simulation, the shape of the protein bound to ergosterol is quite similar to the average structure of the unbound protein at the end of the simulation.

In the simulations of POPI, POPC and POPS, the binding pose of the lipids change significantly. This is not surprising since these ligands are placed inside the cavity based on docking as no crystal structure of these ligands in complex with the protein were available. However, as these phospholipids occupy a large part of the binding cavity, the protein itself does not change conformation as much as seen in the Apo and Ergosterol simulations. In all of the simulations of phospholipids, the alkyl chains of the lipids end up in almost the same apolar part of the protein as the body of ergosterol did. For POPI, the alkyl chains end up in oppositely oriented directions, which could indicate that the exact position of the
alkyl chains is irrelevant for the binding process as long as they are located inside the apolar cavity. This was investigated further by running MD simulations of all the five best docking poses of POPI (Figure S10). As expected, it was found that the exact orientation of lipid tails was largely dependent on the initial binding pose. Interestingly, the polar head group of all phospholipids end up in relatively close proximity to the purple $\alpha$-helix in Figure 6.

To investigate the degree of flexibility of the protein, the root mean square fluctuations (RMSF) of the backbone of the protein determined from five different simulations. The residues of the protein structure are color coded to indicate the position of the residues that differ the most amongst the simulations. b) The protein colored according to a).

Figure 7: a) Root mean square fluctuations (RMSF) of the backbone of the protein determined from five different simulations. The residues of the protein structure are color coded to indicate the position of the residues that differ the most amongst the simulations. b) The protein colored according to a).

(RMSF) of the $\alpha$-carbon atoms of the backbone were calculated based on snapshots from the MD simulations, showing that the most flexible regions are the loops in the green, red and blue region in Figure 7. These regions do not necessarily interact directly with the lipids, as they are largely positioned relatively far from the actual binding site. Instead, high fluctuations could suggest that these segments provide the required flexibility of the protein to accommodate differently shaped lipids. Interestingly, the simulation of the protein in complex with POPC also reflects a high degree of fluctuation in the green and second red loop (7b). This difference between the simulation with POPC and the other phospholipids suggests some lipid specificity in protein conformational changes upon binding of different phospholipids despite their similar size.

To obtain a more visual representation of the correlated movements of the protein, we per-
formed a principal component analysis (PCA) for each of the simulations. In this method, the high-dimensional space of molecular motion variables is reduced by projecting them onto the eigenvectors (principal components) of this vector space, thereby revealing global motions of a protein structure. Projections along the first principal component of NPC2 are visualized in Figure 8. From the PCA, it is clear that the major movements in all of the simulations take place in the loops of the protein (compare Figure 7 and 8). Remarkably, even though the fluctuations in the simulations largely are localized to the same parts of the protein, collective movements of the loops seem very different among the simulations. This is especially true for the red region shown in Figure 7b. The core of the binding pocket is, however, in most cases, relatively conserved. This once again illustrates the point made previously that the loops of the protein are very flexible and likely play a significant role in the protein’s ability to adopt different conformations to accommodate different lipids.

In order to gain further insight into the primary residues that are involved in the binding of the different lipids, we used the MM-PBSA method to calculate the contribution of each residue to the overall binding affinity ($\Delta G$). In this method, the binding free energy is es-

Figure 8: Extreme projections along the first principal component for the NPC2 protein bound to different lipids. The color encodes the position along the first eigenvector as shown with the color-bar.
Figure 9: Contribution of each residue to the binding free energy, $\Delta G$. The energies are calculated using the MM-PBSA method. The segments are colored according to the color-coding in Figure 7b.
timed as the difference between the free energy in the gas phase ($\Delta G_{\text{gas}}$) and the change in solvation energy of the system ($\Delta G_{\text{solv}}$) when the protein binds a lipid. The change in solvation energy is the estimated energy required to repel water from the binding site and around the lipid upon binding. $\Delta G_{\text{gas}}$ can be subdivided into contributions from electrostatic interactions ($\Delta E_{\text{Ele}}$) and from van der Waals interactions ($\Delta E_{\text{vdW}}$). The $\Delta G$ for each residue is presented in Figure 9.

By comparing Figure 7a to Figure 9, a tendency can be observed that those residues contributing the most to the binding free energy are residues that move the least. This, once again, indicates that the residues which make up the binding cavity are the ones that fluctuate the least in the protein. The shape of the binding pocket itself thus seems to be fairly conserved throughout the equilibrated parts of the simulations.

With regard to the binding of ergosterol as a hydrophobic molecule, it is not surprising that the residues contributing the most to the favorable binding of the sterol inside the binding pocket are hydrophobic residues. In fact, 95% of the $\Delta G_{\text{gas}}$ for ergosterol comes from van der Waals interactions (-53.3 kcal/mol of -56.2 kcal/mol). Since van der Waals interactions are generally weak, any individual residue contributes only little to the total free energy of binding. Accordingly, only small per-residue free energy contributions are seen for ergosterol in Figure 9. The high affinity of the NPC2 protein to ergosterol is, thus, mainly due to the ability of the binding cavity to adapt its conformation to the shape of the ligand rather than due to any specific interactions.

In contrast, for the phospholipid ligands, residues could be identified which contribute strongly to the binding affinity, primarily through electrostatic interactions. Most significant is Lys134, which is positively charged at physiological pH. This residue is ideally placed to form ionic interactions with the phosphate group of the phospholipids (Figure 10). Moreover, the aromatic ring of Phe107 is in prime position to form cation-$\pi$ interactions with the positively charged amine/ammonium group of POPC and POPS (Figure 10). These two residues are by far the most stabilizing residues in the simulations of POPC and POPS due to their
charges (POPI does not have a positive charge for cation-π interactions with Phe107). Interestingly, the α-helix in the red segment from residue 109-116 contains three acidic residues. These residues are strongly attracted by the positively charged amine/ammonium group of POPS and POPC through ionic interactions but are at the same time repelled by the phosphate group and, in the case of POPS, the carboxylic acid group. Furthermore, the negatively charged residues are partly desolvated upon binding, which explains the overall destabilizing effect of Asp115 and Glu111 in especially the POPS simulation. Despite the negative effect on the overall estimated binding affinity, we hypothesize that this negatively charged environment, along with the aromatic ring of Phe107, is responsible for the exact positioning of the positively charged amine/ammonium group, while Lys134 is responsible for the positioning of the phosphate group. Thus, the binding of phospholipids is also driven by electrostatic interactions between NPC2 and the charged groups of the phospholipids as opposed to the binding of ergosterol, which is dominated by hydrophobic interactions.

![Figure 10: Visualization of some important residues of NPC2 which are involved in binding of POPC. The structure is from the last snapshot of the simulation.](image)

**Conclusion**

The sterol transfer protein NPC2 has recently been shown to be necessary for ergosterol integration into the vacuolar membrane in yeast. In particular during starvation, NPC2 is
required for vacuolar uptake and processing of lipid droplets and other organelles,\textsuperscript{8} suggesting that this protein plays a more general role in vacuolar lipid digestion. By employing experimental and computational approaches, we show here that yeast NPC2 not only binds sterols but also various phospholipids due to its large binding pocket, which can flexibly adapt to different ligand shapes and sizes. We demonstrate that NPC2 binds analogs of PC, PI, PS and SM with high affinity, while we identified PC and PI as endogenous ligands, apart from ergosterol. Using more sensitive analytical techniques, such as lipid mass spectrometry, additional endogenous ligands will likely be discovered in future experiments.\textsuperscript{46} We show that NPC2 binds phospholipid monomers but can also extract phospholipids from membranes and likely interacts with lipid micelles and other lipid assemblies. It is likely, that by binding to phospholipid monomers NPC2 can prevent formation of phospholipid micelles, which might be an important function of NPC2 as a general lipid solubilizer in the lumen of the yeast vacuole. During lipid hydrolysis, monoacylglycerol phospholipids are generated, which would form micelles and similar small aggregates, unless solubilized by an efficient and abundant lipid transfer protein. Since micelles of amphiphilic substances can damage organelle membranes, micelle formation must be prevented in the vacuolar lumen. Products of lipid hydrolysis, such as monoacylglycerol phospholipids act as weak detergents, whose membrane partitioning is inversely proportional to their CMC.\textsuperscript{47} The ability of NPC2 to extract lipid analogs from membranes could reflect its in vivo function to prevent membrane accumulation of lysophospholipids and thereby membrane damage. In addition, the interaction we found for yeast NPC2 with PS, PC and PI could play a role in recruiting the protein to intravacuolar membranes for efficient sterol pick up, inter-bilayer phospholipid transport and other functions. In fact, binding of both phospholipids and sterols has been described for other membrane-active sterol transfer proteins, such as the oxysterol binding protein Osh4, which binds ergosterol and PI-4-phosphate.\textsuperscript{48} PI-4-phosphate has recently been shown to accumulate at sites of lipophagy, which depends on this particular PI lipid.\textsuperscript{49,50} Future studies are needed to determine, whether yeast NPC2 regulates the abundance of this lipid in the
vacuolar membrane, thereby exerting control over lipohagy and vacuole function. Mammalian NPC2 has been shown to have a smaller binding pocket than yeast NPC2 and is known to bind multiple sterols. Using a nitrocellulose strip binding assay, interaction of mammalian NPC2 with LBPA, PS, PC, PG and PA has been recently demonstrated. Similarly, sterol transfer between membranes by mammalian NPC2 is enhanced in the presence of charged lipids, such as PS, PA and LBPA. Thus, it is likely that mammalian NPC2 also interacts with charged head groups of membrane-embedded phospholipids to fulfill its function as sterol transfer protein in the endo-lysosomes. Whether the affinity of mammalian NPC2 for various phospholipids is sufficient to pick up phospholipid monomers and solubilize phospholipid micelles, as we suggest here for yeast NPC2, warrants future studies.

Acknowledgement

Computations/simulations for the work described herein were supported by the DeIC National HPC Centre, SDU. We acknowledge financial support from the Danish Council for Independent Research (grant agreement No. DFF-7014-00050 and No. DFF-0135-00032), the European Research Council (grant agreement No. 637372), the Carlsberg Foundation (grant agreement No. CF19-0127), and the H2020-MSCA-ITN-2017 COSINE Training network for Computational Spectroscopy In Natural sciences and Engineering (Project ID: 765739).

Uniprot Accession ID for yeast NPC2 used in this study: Q12408

Supporting Information Available

Chemical structures of the fluorescent and spin-labeled lipids used, binding titration curves of DHE, and NBD-tagged PC, SM, or PC-ether to NPC2, and results of fluorescence lifetime fits.
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