Combined 1H-Detected Solid-State NMR Spectroscopy and Electron Cryotomography to Study Membrane Proteins across Resolutions in Native Environments

Baker, Lindsay A; Sinnige, Tessa; Schellenberger, Pascale; de Keyzer, Jeanine; Siebert, C Alistair; Driessen, Arnold J M; Baldus, Marc; Grünewald, Kay

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Combined $^1$H-Detected Solid-State NMR Spectroscopy and Electron Cryotomography to Study Membrane Proteins across Resolutions in Native Environments

Graphical Abstract

Highlights

- CryoET and ssNMR give complementary information about proteins in native membranes
- One sample can be prepared for both methods without the use of detergents
- Hybrid method shows differences between purified and native preparations of YidC
- Sample preparation reduces costs and time and suggests new strategy for assignment

Authors

Lindsay A. Baker, Tessa Sinnige, Pascale Schellenberger, ..., Arnold J.M. Driessen, Marc Baldus, Kay Grünwald

Correspondence

lindsay@strubi.ox.ac.uk (L.A.B.), m.baldus@uu.nl (M.B.), kay@strubi.ox.ac.uk (K.G.)

In Brief

Membrane proteins are often treated with detergents, which can affect structure and activity. Baker et al. apply a hybrid method to bacterial membrane proteins in native membranes without detergent, using solid-state NMR spectroscopy and electron cryotomography. They find that the structure and function of YidC differ with and without detergent.
Combined \(^{1}\text{H}\)-Detected Solid-State NMR Spectroscopy and Electron Cryotomography to Study Membrane Proteins across Resolutions in Native Environments

Lindsay A. Baker,1,2,9,* Tessa Sinnige,1,8 Pascale Schellenberger,2,6 Jeanine de Keyzer,3,4,7 C. Alistair Siebert,2,8 Arnold J.M. Driessen,3,4 Marc Baldus,1,* and Kay Grünwald2,*

1NMR Spectroscopy, Bijvoet Center for Biomolecular Research, Utrecht University, 3584 CH Utrecht, the Netherlands
2Oxford Particle Imaging Centre, Division of Structural Biology, University of Oxford, The Wellcome Trust Centre for Human Genetics, Roosevelt Drive, Oxford OX3 7BN, UK
3Department of Molecular Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 7, 9747 AG Groningen, the Netherlands
4The Zernike Institute for Advanced Materials, University of Groningen, Nijenborgh 11, 9747 AG Groningen, the Netherlands
5Present address: Centre for Misfolding Diseases, Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, UK
6Present address: School of Life Sciences, University of Sussex, Brighton BN1 9QG, UK
7Present address: Hanze University of Applied Sciences, Zernikeplein 7, 9747 AS Groningen, the Netherlands
8Present address: Electron Bio-Imaging Centre, Diamond Light Source, Harwell Science & Innovation Campus, Didcot, Oxfordshire OX11 0DE, UK
9Lead Contact
*Correspondence: lindsay@strubi.ox.ac.uk (L.A.B.), m.baldus@uu.nl (M.B.), kay@strubi.ox.ac.uk (K.G.)
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SUMMARY

Membrane proteins remain challenging targets for structural biology, despite much effort, as their native environment is heterogeneous and complex. Most methods rely on detergents to extract membrane proteins from their native environment, but this removal can significantly alter the structure and function of these proteins. Here, we overcome these challenges with a hybrid method to study membrane proteins in their native membranes, combining high-resolution solid-state nuclear magnetic resonance spectroscopy and electron cryotomography using the same sample. Our method allows the structure and function of membrane proteins to be studied in their native environments, across different spatial and temporal resolutions, and the combination is more powerful than each technique individually. We use the method to demonstrate that the bacterial membrane protein YidC adopts a different conformation in native membranes and that substrate binding to YidC in these native membranes differs from purified and reconstituted systems.

INTRODUCTION

Cellular organization relies on compartmentalization by lipid membranes, around which cells install protein networks that establish further function. These membrane proteins are challenging to characterize, as their native environment is complex and heterogeneous. Despite significant work with membrane proteins, the rate at which new structural information is being produced has decreased since 2005 (White, 2016). Most structural techniques rely on purification of membrane proteins using detergents, followed in some cases by reconstitution into synthetic lipid bilayers. Neither system can fully mimic the complex nature of these proteins’ natural environment, and the choice of mimetic can have significant impact on both structure and function (Zhou and Cross, 2013). Purification can also disrupt higher-order structure, including oligomerization (Mi et al., 2008), complex formation (Sychev et al., 1996), or metabolic organization (Schagger and Pfeiffer, 2000). Therefore, it is desirable to be able to study membrane proteins and their structure in their native membranes. Two methods that allow for structural investigations in native membranes are solid-state nuclear magnetic resonance spectroscopy (ssNMR) and electron cryotomography (cryoET).

CryoET and ssNMR provide highly complementary information. CryoET involves imaging individual events, with each molecule potentially in a different state, while ssNMR uses bulk measurements. Motion and dynamics are implicitly recorded in ssNMR experiments from nanosecond to millisecond time scales, while cryoET is limited by the speed at which samples can be vitrified, and is therefore useful for snapshots of biological processes on the seconds-to-hours timescale. Nanometer-scale spatial information is intrinsic to cryoET, while ssNMR is much more sensitive to chemical information on the Ångstrom scale. Furthermore, ssNMR exploits isotope labeling to exclude background signals, whereas cryoET records the full environment, providing macromolecular context for measurements. CryoET and ssNMR have both been used successfully to study fibrillar structures (for a review, see Cuniasse et al., 2017) and, more recently, microtubule-protein...
Another area of development is in 1H-detected spectral sensitivity for such samples (Jacso et al., 2012; Kaplan et al., 2015; Renault et al., 2012a) or mammalian membrane proteins embedded in their natural plasma membranes (Kaplan et al., 2016a, 2016b). Recent methodological advancements in Dynamic Nuclear Polarization have improved spectral sensitivity for such samples (Jacso et al., 2012; Kaplan et al., 2015, 2016a, 2016b; Renault et al., 2012b; Yamamoto et al., 2015). Another area of development is in 1H-detected MAS ssNMR experiments, where the higher gyromagnetic ratio of protons can enhance overall spectroscopic sensitivity provided that MAS spinning rates >40 kHz are used (Andreas et al., 2010; Asami and Reif, 2013; Medeiros-Silva et al., 2016; Sinnige et al., 2014; Ward et al., 2011). With faster spinning, line widths are generally narrower; sample preparation and choice of labels can improve spectral resolution (Andreas et al., 2010; Asami and Reif, 2013; Fricke et al., 2017; Medeiros-Silva et al., 2016; Sinnige et al., 2014; Ward et al., 2011).

CryoET has been used to study a wide range of samples, from purified protein complexes to intact viruses, bacteria, and eukaryotic cells, preserved in a frozen, hydrated state that mimics physiological conditions. Briefly, a series of projection images of the same specimen is collected with different orientations relative to the electron beam, followed by computational processing to recover three-dimensional structural information without averaging (for a recent review, see Beck and Baumeister, 2016). As the sample and stage thickness prevent tilting to 90°, there is a “missing wedge” of information in Fourier space. This missing information can be compensated for by averaging together three-dimensional subvolumes extracted from tomograms, which are differentially oriented relative to the missing wedge. CryoET (and other forms of cryoEM) also recently benefited from technological advancements. In particular, direct electron detectors have significantly increased the signal in images (McMullan et al., 2014). Some recent examples of bacterial systems studied by cryoET include work investigating the organization of the pilus in Myxococcus xanthus (Chang et al., 2016), the injection of pathogenic factors into host cells by Chlamydia trachomatis (Nans et al., 2015), and the formation of cellular structures organizing DNA replication during phage infection (Chaikeeratisak et al., 2017).

To take full advantage of the complementarity between ssNMR and cryoET, and recent technological improvements in 1H detection and direct detectors, respectively, we set out to create a sample preparation method for the structural and functional study of membrane proteins in their native environment, where the same specimens could be used for both techniques. To maintain the native membrane environment, we avoided altogether the use of detergents or other extraction strategies. These samples also needed to balance the sensitivity of 1H-detected ssNMR experiments with reasonable protein expression levels to avoid excess disruption to the membrane environment. As structure is tightly linked to function, accessibility to the membrane surfaces for functional or binding assays was also an important consideration. Similarly, membrane morphologies needed to be reflective of, e.g., native cell envelope ultrastructure. Furthermore, a range of orientations is desirable to compensate for the missing wedge in cryoET.
Here, we present a combined 1H-detected ssNMR and cryoET investigation of the structure, function, and native environment of YidC in *Escherichia coli*. YidC is an inner membrane protein that helps fold and insert other inner membrane proteins (Scotti et al., 2000). YidC has also been shown to insert some substrates, such as subunit c of ATP synthase, independently of the Sec translocon system (van der Laan et al., 2004). *E. coli* ribosomes with substrate membrane protein nascent chains (RNCs) bind and insert substrate via purified and reconstituted YidC (Kedrov et al., 2013). The structure of purified YidC was determined in the lipidic cubic phase by X-ray crystallography (Kumazaki et al., 2014) and also, at lower resolution, in nanodiscs bound to RNCs by single-particle cryoEM (Kedrov et al., 2016). YidC can be produced with high yield after overexpression in *E. coli*, allowing for purified and reconstituted control samples (Baker et al., 2015), and binding of ribosomes to YidC has been shown to differ depending on the membrane mimetic used (Kedrov et al., 2013). We use our hybrid method to show that the conformation and likely dynamics of YidC in native membranes differs from purified and reconstituted YidC, and observe corresponding RNC binding differences.

RESULTS

Single Sample Preparation for Hybrid Methods

A key goal of this work was to develop a single sample preparation strategy for both ssNMR and cryoET. To achieve the desired sensitivity and resolution by NMR spectroscopy requires incorporation of NMR-active nuclei (1H, 13C, and 15N) into the molecule of interest. To selectively incorporate the isotopes while maintaining an NMR-silent 2H12C14N cell envelope background, we used the antibiotic rifampicin to inhibit the native *E. coli* RNA polymerase. The gene of interest then was transcribed by the T7 polymerase, as described previously (Baker et al., 2015). Rifampicin-treated cell envelopes have negligible background protein signal when measured by ssNMR (Baker et al., 2015). After rifampicin treatment and protein expression, cell envelopes were harvested by gentle cell lysis and ultracentrifugation. For cryoET, the cell envelopes were diluted 1 in 300 in buffer before vitrification on a carbon-coated grid. An overview of the method is presented in Figure 1.

To confirm that rifampicin treatment had not changed cellular or membrane structure, we first used cryoEM to investigate the morphology of the *E. coli* cells and cell envelope samples prepared by this method. Small samples of the cultures were taken immediately before cell envelope harvesting and vitrified without any further treatment. Many cells exhibited typical size and shape (rods of ~2 μm by 1 μm), but some cells (~20%) were longer (between 3 and 12 μm) and a small number (<10%) were rounded or showed evidence of lysis (Figure S1). The appearance of the cell envelope samples also remained consistent, with or without rifampicin treatment and heterologous

![Figure 2. The Morphology of Cell Envelope Samples by cryoEM](image-url)

(A) Cell envelopes were vitrified on graphene oxide-treated holey carbon grids. The membranes adopt a variety of shapes and sizes, with many maintaining a native ultrastructure.

(B) Membrane structures of different shapes and sizes maintain the native cell wall architecture when viewed at higher magnification of outer membrane (black line), cell wall (red line), and inner membrane (white line). When the inner and outer membranes separate (black arrowheads), the cell wall appears to favor the outer membranes.

See also Figures S1 and S2.
Probing Protein Conformation and Dynamics with ssNMR of Cell Envelope Samples

Satisfied that the YidC cell envelopes maintained a native-like ultrastructure, we then subjected them to 1H-detected MAS ssNMR. For comparison, we used a conventional sample of YidC purified from the same E. coli strain and reconstituted into E. coli lipids, as described previously (Baker et al., 2015). While 1 L of 15N13C2H culture was required to fill a 1.3-mm MAS rotor with purified and reconstituted sample, only 20 mL was required for the cell envelope sample, reducing costs by approximately 50-fold. At 55 kHz MAS and sample temperatures around 30°C, we saw no degradation in cell envelope samples treated with 2 mM EDTA for approximately 2 weeks. A two-dimensional [15N,1H] proton-detected Hartmann-Hahn cross-polarization (CP) experiment (Hartmann and Hahn, 1962) run on both cell envelopes and a purified and reconstituted sample is shown in Figure 3. This experiment took approximately 2–3 hr to run on an 800-MHz spectrometer for both samples; comparison of projected intensities normalized for the number of scans suggests that the concentration of YidC is about 2-fold lower in the cell envelope samples (Figure S3B). As these samples were grown in 2H2O and then washed in 1H2O, only water-exchangeable protons (e.g., amide protons) will be present. Although the spectra agree in many places, there are significant differences between the native and reconstituted samples: ~18 resolvable peaks shift (mean absolute shifts of 0.05 and 0.4 ppm in 1H and 15N, respectively) and >35 intensities change (stars) in the purified sample, relative to the cell envelope, suggesting that the structure and likely dynamics of YidC are different in native membranes. See also Figure S3.

Figure 3. Overlay of 1H-detected (CP-based) 15N-1H correlation spectra of YidC in Cell Envelopes (Green) and Purified and Reconstituted in E. coli Phospholipids (Gray) at 55 kHz MAS

Although overall the spectra agree well (>30 resolved peaks remain unchanged), 18 resolved peaks move (arrows) and >35 intensities change (stars) in the purified sample, relative to the cell envelope, suggesting that the structure and likely dynamics of YidC are different in native membranes. See also Figure S3.

To understand these changes in structure and likely dynamics, assignment of the peaks in the spectra to specific atoms and residues in the protein is necessary. Routine assignment of larger α-helical membrane proteins remains challenging, and there are no NMR assignments available for YidC. Unfortunately, the limited agreement of chemical shift predictions precludes using the crystal structure of purified YidC as a basis for assignment (Figure S3). Significant progress has been made in obtaining ssNMR 13C and 15N resonance assignments for α-helical membrane proteins up to about half of the size of YidC (Cady et al., 2010; Etzkorn et al., 2007; Park et al., 2012; Sharma et al., 2010; Wang et al., 2013). Combined amino acid-specific labeling with 1H ssNMR (Sinnige et al., 2014) and fractional proton labeling (Mance et al., 2015; Medeiros-Silva et al., 2016) has been used to reduce spectral complexity, but this approach is not cost effective for samples requiring large sample volumes. However, given the 50-fold reduction in sample volume for cell envelope samples, we decided to investigate amino acid-specific labeling for YidC. YidC has an extended cytoplasmic domain, which was recently shown to adopt an unusual conformation (Kumazaki et al., 2014). We therefore chose to label methionine, arginine, and lysine residues, which are prevalent in this region (Figure 4A), and avoid unintentional labeling of additional amino acids via metabolic scrambling. Out of 548 amino acids in YidC, 22 are methionine (4%), 29 are lysine (5.3%), and 15 are arginine (2.7%). One sample was prepared with methionine and arginine labeled (6.7% of total residues labeled), and another with methionine and lysine labeled (9.3% of total residues labeled). Simply by comparing two-dimensional these samples (Figures 4B–3D), we tentatively identified 10 peaks as arginine (out of 15 total),
19 as lysine (29 total), and 17 as methionine (22 total), demonstrating the feasibility and potential of this method. Further, the number of peaks we have identified suggests that we are observing resonances from each domain in YidC (periplasmic, transmembrane, and cytoplasmic). With improved pulse sequences for three- or higher-dimensional magnetization transfer (see, e.g., Andreas et al., 2010; Fricke et al., 2017; Ward et al., 2015b) in dynamic, heterogeneous samples such as cell envelopes, we anticipate that it will be possible to use pairwise labeling of specific amino acids to assign large α-helical membrane proteins.

Figure 4. 1H-detected 15N-1H Correlation Spectra of Amino Acid Specifically Labeled YidC

(A) The positions of methionine (purple), arginine (red), and lysine (blue) in the crystal structure of YidC from E. coli (PDB: 3WVF; Kumazaki et al., 2014), used to choose the labeling scheme for 15N-1H CP spectra of amino acid-labeled YidC in cell envelopes. (B) Overlay of specifically labeled spectra: red, 15N-1H methionine and arginine; blue, 15N-1H methionine and lysine. These amino acids are not used by E. coli in the metabolic production of other amino acids. By comparing the two samples, putative amino acid types (triangles, methionine; squares, arginine; circles, lysine) can be ascribed based on their presence in one (lysine or arginine, depending on sample) or both (methionine), as shown on each spectrum. (C and D) Overlay of fully labeled YidC cell envelopes (gray) with (C) 15N-1H methionine and arginine from (B) (red) and (D) 15N-1H methionine and lysine from (B) (blue). All correlations seen in specifically labeled samples appear also in fully labeled YidC at lower contour levels.

Structural Characterization of Ribosomes with Nascent Chains Binding to YidC in Cell Envelope Samples by CryoET and Subvolume Averaging

YidC interacts with translating ribosomes whose nascent chain is a YidC substrate (Kedrov et al., 2013). We therefore overexpressed subunit c of ATP synthase, a YidC substrate (van der Laan et al., 2004), with an added SecM stall sequence and a strep tag in E. coli cells lacking the chaperone Trigger Factor, and purified RNCs by affinity chromatography, as described previously (Wu et al., 2012). We then incubated the RNCs with cell envelopes containing overexpressed YidC before vitrification for cryoET. Slices through an example tomogram are shown in Figure 5A. RNCs can be observed in proximity to membranes (Figures 5B and 5C). Ribosomes are known to interact with the air-water interface (Frank et al., 1991; Kedrov et al., 2016), which is typically overcome by adsorbing the ribosomes to a thin carbon film before vitrification (Frank et al., 1991), or, as recently proposed, by the addition of small amounts of detergents in the buffer (Kedrov et al., 2016). However, for our samples, adsorption to the carbon film would be as disruptive as attraction to the air-water interface, and detergent could perturb the cell envelopes, so we chose to consider only RNCs not found at the air-water interface. Of these distinct ribosomes, ~42% were in proximity to a membrane (Figure 5E).

To investigate the interaction of ribosomes with nascent chains and these membranes, we used subvolume averaging. A total of 396 subvolumes were extracted from 32 tomograms, and aligned to an initial average generated by defining the membrane normal. After several rounds of alignment, the subvolumes were classified using principal component analysis and k-means clustering on the ribosome region of the subvolumes only. Although of low resolution, the approximate position for the
DISCUSSION

We have established a framework for a hybrid method to probe the structure, function, and native environment of membrane proteins in native bacterial membranes using $^1$H-detected ssNMR spectroscopy and cryoET. The combination of the two methods provides complementary information and is more powerful than each method individually. Our ssNMR data are compatible with conformational and possibly dynamical changes in YidC when comparing the native environment and reconstituted samples. In parallel, by cryoET, we observed that binding of RNCs of F$_2$C to YidC is different in native membranes than has been published previously for reconstituted YidC (Kedrov et al., 2016). These authors measured dissociation constants of 85 nM and 210 nM for RNC binding to YidC by fluorescence correlation spectroscopy in nanodiscs with different lipid compositions. Based on these dissociation constants, we would expect very high levels of observed RNC binding (98.9% and 97.5%, respectively) for the conditions found in the native membrane preparations here (2.5 $\mu$M [total YidC] and 10 $\mu$M [total RNC]). The role of crowding or external binding partners present in the native membranes, effects of lipid composition on RNC interactions, or our observed structural changes in YidC itself may underlie the lower binding rates for native membranes. For example, a 3-fold increase in RNC-YidC K$_D$ was observed in the absence of DOPE (dioleoylphosphatidylethanolamine) (Kedrov et al., 2013), and the glycolipid MPIase has been proposed to modulate YidC activity (Nishikawa et al., 2017). The function of YidC suggests that less stable binding in a native environment might actually be beneficial in cells, preventing buildup of stalled ribosomes or unfolded membrane proteins and ensuring a supply of available YidC.
With native membrane preparations, by removing the use of detergent in any step of the process the biological environment of membrane proteins is maintained. We have shown that our cell envelope preparations adopt a variety of morphologies, with many maintaining the native structure of the two bilayers with a cell wall. Working with these native membranes can pose unique challenges: methods such as sonication, freeze-thawing, or extrusion, typically used for synthetic bilayers, produce different membrane morphologies when applied to cell envelopes. However, as the purification procedure is reduced substantially, the amount of time and material needed is correspondingly reduced. From transformation to the final sample, cell envelopes can be prepared in 4 days, while to purify and reconstitute often requires 2 weeks. Close to 1 L of isotopically labeled culture is needed to purify enough protein to fill a 1.3-mm MAS rotor; cell envelope samples can be grown in 20 mL, producing a significant (~50-fold) reduction in costs for isotopes with only an approximately 2-fold decrease in protein concentration. This reduction in costs opens up a new possibility for assignment of large membrane proteins by MAS ssNMR. Direct detection of protons with faster spinning speeds produces sufficient signal to observe even sparsely labeled membrane proteins in the cell envelope. Therefore, we propose that several samples, strategically labeling combinations of two or three amino acids per sample, in combination with three- or higher-dimensional experiments, provides an approach toward assignment of residues in large membrane proteins by ssNMR. This approach is significantly less intensive than mutagenesis, and can be tailored to individual proteins or domains through the choice of amino acids. Here, we have chosen to use amino acids that are not further metabolically processed in E. coli (Hullo et al., 2007; Jensen and Wendisch, 2013; Kaneshisa et al., 2017). To avoid unintended labeling of amino acids via metabolic reprocessing where this is not the case (for example, aspartate or glutamate), 1H13C NMR-silent amino acids could be added in addition to those 15N14C amino acids to be observed in a reverse labeling strategy (Etzkorn et al., 2007; Heise et al., 2005; Umbarger and Brown, 1998; Vuister et al., 1994). Alternatively, strategically selected pairs of amino acids, where one amino acid feeds into the synthesis pathways of a second amino acid, could be labeled together. The method presented here is generally applicable to E. coli membrane proteins and has the potential to be expanded to other bacterial and eukaryotic cell lines. Many bacteria are susceptible to rifampicin; a similar strategy could be used in eukaryotic cells with MazEF-based destruction of native mRNA for isotope labeling (Suzuki et al., 2005) and focused-ion beam milling to thin cells for electron tomography (Rigort et al., 2012). Future work could also focus on reducing the overexpression of the protein of interest to lower levels, more carefully mimicking the endogenous expression. Site-specific dynamic nuclear polarization could be used to increase signals in ssNMR (van der Cuijisen et al., 2015; Kaushik et al., 2016; Rogawski et al., 2017), and high-precision correlative light and electron microscopy (Schellenberger et al., 2014) could be used to locate rare events of interest in cryoET.

Conclusions
We have demonstrated a sample preparation that maintains the native environment of membrane proteins and is suitable for both ssNMR and cryoET. The use of detergents or other membrane-extraction strategies is avoided entirely, and the method can be used to probe function and binding assays with membranes reflective of the native cellular ultrastructure. By minimizing interventions, sample costs have been reduced by more than an order of magnitude, with a 3-fold decrease in preparation time. Combining cryoET and ssNMR provides complementary information: conformational and dynamical changes observed by ssNMR help interpret functional results from cryoET. This approach provides an attractive framework through which to effectively characterize the structure, dynamics, and function of membrane proteins.

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SUPPLEMENTAL INFORMATION
Supplemental Information includes four figures and can be found with this article online at https://doi.org/10.1016/j.str.2017.11.011.

AUTHOR CONTRIBUTIONS

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REFERENCES


STAR METHODS

KEY RESOURCES TABLE

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<td><a href="http://bio3d.colorado.edu/PEET/">http://bio3d.colorado.edu/PEET/</a></td>
</tr>
<tr>
<td>Chimera</td>
<td>(Pettersen et al., 2004)</td>
<td><a href="https://www.cgl.ucsf.edu/chimera/">https://www.cgl.ucsf.edu/chimera/</a></td>
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<td>Topspin</td>
<td>Bruker Biospin</td>
<td><a href="https://www.bruker.com/service/support-upgrades/software-downloads/nmr.html">https://www.bruker.com/service/support-upgrades/software-downloads/nmr.html</a></td>
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<td>Sparky</td>
<td>(Goddard and Kneller, 2008)</td>
<td><a href="https://www.cgl.ucsf.edu/home/sparky/">https://www.cgl.ucsf.edu/home/sparky/</a></td>
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<td>SerialEM</td>
<td>(Mastronarde, 2005)</td>
<td><a href="http://bio3d.colorado.edu/SerialEM/">http://bio3d.colorado.edu/SerialEM/</a></td>
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<td>Tomography</td>
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<td><a href="https://www.fei.com/software/tomography-4/">https://www.fei.com/software/tomography-4/</a></td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
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<td>Quantifoil holey carbon copper grids</td>
<td>Quantifoil Micro Tools GmbH</td>
<td>Cu 200 R2/1</td>
</tr>
<tr>
<td>Graphene oxide</td>
<td>Sigma-Aldrich</td>
<td>763705</td>
</tr>
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</table>

CONTACT FOR REAGENTS AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Lindsay Baker (lindsay@strubi.ox.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

*Escherichia coli* LEMO (New England Biolabs) was cultured in LB (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, pH 7.0) or M9 minimal medium (6 g/L Na2HPO4, 3 g/L KH2PO4, 0.5 g/L NaCl, 1 g/L NH4Cl, 5 g/L glucose, 5.5 mg/L thiamine, 0.01 mM...
FeSO₄; 2 mM MgSO₄; 0.01 mM CaCl₂; micronutrients: 0.003 μM ammonium molybdate, 0.4 μM boric acid, 0.03 μM cobalt chloride, 0.01 μM copper sulphate, 0.08 μM manganese chloride, 0.01 μM zinc chloride; and vitamins: 1 mg/L D-biotin, 0.5 mg/L choline chloride, 0.5 mg/L folic acid, 1 mg/L myoinositol, 0.5 mg/L nicotinamide, 0.5 mg/L pantothenic acid, 0.5 mg/L pyridoxal HCl, 0.05 mg/L riboflavin) with 35 μg/ml chloramphenicol at 37 °C unless otherwise specified. Single colonies were selected on LB agar, and liquid cultures were grown with 250 rpm shaking.

**METHOD DETAILS**

Protein Expression and Cell Envelope Isolation

A previously described plasmid (Baker et al., 2015) carrying the gene yidC from *Escherichia coli* (UniProt ID: P25714) with a N-terminal 6xHis tag under a T7 promoter was used to transform *E. coli* LEMO cells (New England Biolabs) by heat shock before plating on LB with ampicillin (AMP) at 50 μg/ml and chloramphenicol (CAM) at 35 μg/ml. Precultures were grown in LB with AMP and CAM and then transferred to the M9 minimal media described above (Folkers et al., 2004) with 1.0 g/L 14NH₄Cl, 5.0 g/L 13C₂H-glucose, AMP and CAM overnight. Between 50 and 500 mL volumes of M9, supplemented as above but with 13C₂H-glucose and prepared in D₂O, were inoculated with the overnight cultures to OD 0.1, and grown at 37 °C with shaking until reaching an OD ~ 1.5 - 2.0. Cells were harvested by centrifugation at 4,000 x g for 15 minutes before resuspension in equal volumes isotoically labeled supplemented M9 (1.0 g/L 15NH₄Cl, 5.0 g/L 13C₂H-glucose for uniformly labeled samples in D₂O; 1.0 g/L 14NH₄Cl, 5.0 g/L 12C₂H-glucose and 200 mg/L each 1H, 15N and 13C labeled amino acid in D₂O for specifically labeled samples). IPTG (isopropyl β-D-1-thiogalactopyranoside) at 0.5-1 mM final concentration was added to induce expression of the T7 polymerase and the cultures were incubated at 28 °C for 30 min with shaking. Rifampicin was added to a final concentration of 200 μg/mL and the cultures were incubated with shaking in the dark at 25 °C overnight. Cells were harvested by centrifugation at 4,000 x g for 10 min at 4 °C, and resuspended in 10 mL cold lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl in H₂O). Cells were lysed in a pressure cell homogenizer (Constant Systems) at 8,000 psi without the addition of lysozyme. Cell debris was removed by centrifugation at 4,000 x g for 10 min. Membranes were harvested by centrifugation at 100,000 x g for 1 hr.

Purified and Reconstituted Sample Preparation

Reference YidC proteoliposome samples were prepared in a similar manner to the cell envelope samples, as described previously (Baker et al., 2015). Briefly, after preculture, the main culture was inoculated in M9 in D₂O with 13C₂H-glucose and 15NH₄Cl at OD 0.1, and grown to OD 0.6 prior to induction with IPTG and 10 μM final concentration rhamnose. Cells were grown at 37 °C until the OD was greater than 2.0, then pelleted and lysed, and the membranes isolated as described for the cell envelope samples. Membranes were solubilized at 4 °C by stirring with 2% (w/v) dodecyl maltoside (DDM) in 10 mM phosphate buffer (pH 6.8) containing 250 mM NaCl, 2 mM 2-mercaptoethanol, 10 % (v/v) glycerol, 0.03 % (w/v) DDM and 20 mM imidazole (Buffer A) for 2 hours. Insoluble material was pelleted at 100,000 x g for 1 hr, and the supernatant was bound to 5 mL Ni-NTA resin (Qiagen) overnight. The resin was washed with 4 column volumes of buffer A, and then with 2 CV of buffer B (10 mM phosphate buffer pH 6.8, 100 mM NaCl, 2 mM 2-mercaptoethanol, 10 % (v/v) glycerol, 0.03 % (w/v) DDM and 20 mM imidazole). YidC was eluted with 5 CV of buffer B with 380 mM additional imidazole. The eluent was dialyzed four times for ~2 hrs against 75 mL of Buffer B without imidazole. The protein concentration was estimated using the BCA assay (Pierce) and then mixed with *E. coli* polar lipid extract (Avanti Polar Lipids), dissolved in water, at a ratio of 2 mg YidC: 1 mg lipid. DDM was removed by overnight incubation with BioBeads (BioRad) and YidC proteoliposomes were harvested by centrifugation at 100,000 x g for 1 hour. The proteoliposomes were washed in 10 mM phosphate buffer, pH 6.8, and pelleted at 125,000 x g for ~2hrs before being packed into a 1.3 mm MAS rotor.

Spectroscopy, Processing, and Referencing

Membranes were washed twice with 10 mM phosphate buffer pH 6.8 (in H₂O) to remove any Tris from the lysis buffer and collected by centrifugation at 125,000 x g for 1 ~ 2 hrs before packing into a 1.3 mm rotor for magic angle spinning (MAS). YidC samples were measured on a 800 MHz wide-bore or 700 MHz narrow-bore spectrometer (Bruker Biospin, Germany) with a 1.3 mm 1H, 13C, 15N MAS probe at 55 kHz MAS frequency, and with a set temperature of 253 K (corresponding to an effective temperature of ~ 30 °C). Spectra were referenced against adamantane (Harris et al., 2008) and histidine (Wei et al., 1999) powders. Data were processed with TopSpin 3.0 (Bruker Biospin) and analyzed using Sparky (Goddard and Kneller, 2008). Chemical shift predictions were made with Shiftx2 (Han et al., 2011) and FANDAS (Gradmann et al., 2012) using atomic model PDB ID 3wvf (Kumazaki et al., 2014).

RNC Complex Purification and Cell Envelope Binding

Ribosomes with F₉C nascent chains (RNCs) were prepared as described previously (Wu et al., 2012). Briefly, F₉C with a SecM stall sequence and strep tag was over-expressed in an *E. coli* strain lacking the chaperone Trigger Factor. Cells were cooled on ice and harvested by centrifugation, before repeated cycles of freeze-thaw to lyse. Cell debris was removed by centrifugation at 4000xg for 10 min and RNCs were purified by affinity chromatography using streptactin beads (IBA Bioscience), eluted with biotin, and concentrated to 2 μM with centrifugal concentrators (Millipore) before snap freezing in liquid nitrogen and storage at -80 °C in aliquots until further use. For RNC binding experiments, membranes were diluted 1:30 in 50 mM Tris-HCl pH 7.4, 150 mM KCl, 10 mM MgCl₂ before 10 ul was mixed with 10 ul of RNC and incubated on ice for 1 hr. The mixture was diluted 1:10 in the same buffer before
vitrifying as described for cell envelopes without RNCs below. Final concentrations of RNCs and YidC were estimated using the tomogram volumes and assuming a uniform distribution of YidC in the *E. coli* inner membrane.

**Electron Cryotomography and Image Processing**

Cell envelopes were diluted 1:300 from the ssNMR samples in lysis buffer before application to Quantifoil 2/1 holey carbon grids with or without the addition of graphene oxide. Graphene oxide grids were prepared immediately before use by applying 2 ul of a 2 mg/ml solution of graphene oxide sheets (Sigma) to a freshly glow-discharged grid for 30 s, blotting away excess, and washing three times in water. Samples were incubated on grids for 10 s, before for blotting for 8 s by hand and plunging into a bath of propane/ethane with a manual plunger. Grids were stored under liquid nitrogen until imaging. Electron microscopy data was recorded on either a TF30, TF30 Polara or Titan Krios microscope (FEI), equipped with K2 direct electron detectors and Quantum energy filters (Gatan). Tomographic data was collected with SerialEM (Mastronarde, 2005) or FEI Tomography, with pixel sizes between 2 and 3 Å/pixel at the specimen level and the energy selecting slit set to 20 eV, with a collection scheme of ±45° tilts (starting from 0° up, and returning to 0° to collect the negative tilts) with a 3° tilt interval. 7-10 image frames (0.2 s exposure/frame) in counting mode were collected per tilt at a dose rate of ~5e-/unbinned pix/sec, giving an overall dose of ~ 60 e/Å². Frames were aligned and filtered for radiation damage using Unblur (Grant and Grigorieff, 2015). Tomograms were reconstructed using IMOD (Kremer et al., 1996). Contrast transfer functions were measured and data phase-flipped in IMOD. Tomograms were further processed for viewing in FIJI (Schindelin et al., 2012). Subvolumes were picked manually in IMOD and subvolume averaging and classification was done with PEET (Heumann et al., 2011). Subvolume averages and atomic models were visualised with USCF Chimera (Pettersen et al., 2004).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Subvolume averaging and classification was done using principal component analysis and k-means clustering as implemented in PEET. The number of subvolumes in the classes of undetermined RNCs were used as uncertainty ranges in the RNC binding measurements.

**DATA AND SOFTWARE AVAILABILITY**

The density maps corresponding to the average and 6 classes of RNCs bound to the native YidC membranes have been deposited in the Electron Microscopy Data Bank (EMDB) under ID codes 3909, 3919, 3920, 3921, 3922, 3923, and 3924.