Biochimica et Biophysica Acta 1833 (2013) 2781–2788

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Post-translational membrane insertion of an endogenous YidC substrate

Philip J. Robinson, Cheryl A. Woolhead *

Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK

ARTICLE INFO

Article history:
Received 17 May 2013
Received in revised form 27 June 2013
Accepted 8 July 2013
Available online 18 July 2013

Keywords:
YidC
Membrane insertion
Reconstitution
Targeting
F_0c

ABSTRACT

Membrane protein insertion is controlled by proteinaceous factors embedded in the lipid bilayer. Bacterial inner membrane proteins utilise the Sec translocon as the major facilitator of insertion; however some proteins are Sec independent and instead require only YidC. A common feature of YidC substrates is the exposure of a signal anchor sequence when translation is close to completion; this allows minimal time for targeting and favours a post-translational insertion mechanism. Despite this there is little evidence of YidC's post-translational activity. Here we develop an experimental system that uncouples translation and insertion of the endogenous YidC substrate F_0c (subunit c of the F_0F_1 ATP synthase). In this process we (i) develop a novel one step purification method for YidC, including an on column membrane reconstitution, (ii) isolate a soluble form of F_0c and (iii) show that incubation of F_0c with YidC proteoliposomes results in a high level of membrane integration. Conformational analyses of inserted F_0c through Blue Native PAGE and fluorescence quenching reveal a native, oligomerised structure. These data show that YidC can act as a post-translational insertase, a finding which could explain the absence of a ribosome binding domain on YidC. This correlates with the post-translational activity of other YidC family members lacking the ribosome binding domain.

1. Introduction

Membrane proteins synthesised on cytosolic ribosomes insert into specific target lipid bilayers to form native, functional three dimensional structures. In bacteria, most integral inner membrane proteins are composed of hydrophobic α-helical bundles that span the membrane [1]. During targeting transmembrane domains are transferred from the hydrophilic environment of the cytosol into the hydrophobic membrane core; this process requires complexes that control and catalyse insertion. The majority of membrane proteins utilise the Sec system as the major facilitator of insertion [2] but some membrane proteins are Sec independent [3]. These substrates tend to be short hydrophobic proteins with one or two transmembrane spanning domains and short hydrophilic loops [4]; however larger substrates have more recently been identified [5]. Originally it was believed that these small proteins could insert spontaneously [6]. However it was later discovered that a membrane protein called YidC provides an alternative insertion pathway [7]. YidC is essential for cell survival [8] and more abundant than the Sec translocon with an estimated 2,700 copies of YidC per cell [9] compared to 300–600 copies of the Sec translocon [10]. Despite the importance of YidC, its mechanism and precise function have been difficult to define. It has been shown that YidC has chaperone activity [11], can insert specific substrates [7], and has a supportive role in the Sec system [12].

The bacteriophage procoat proteins M13 and Pf3 were the first identified YidC dependent membrane proteins [8,13]. Later the c-subunit of the F_0 component of the bacterial ATP synthase (F_0c) was identified as the first endogenous YidC substrate [14–16]. F_0c oligomerises into a decamer to form the membrane spanning component of the F_0F_1 ATP synthase. Like all membrane proteins, YidC dependent substrates must target to specific membranes before insertion. It is unclear if YidC substrates utilise the same SRP targeting machinery that is required for Sec dependent insertion or if they have an alternative targeting mechanism, as different studies have reported a variation in SRP dependence between different substrates and experimental systems [17]. Furthermore it is still unknown how YidC substrates are discriminated from Sec substrates to direct insertion through the YidC pathway in preference to the Sec pathway.

Previously we have studied co-translational folding events in the ribosome exit tunnel utilising F_0c as a model substrate [18]. We found that nascent chain compaction occurs in the tunnel before an SRP interaction is detected at which stage synthesis is ~10 amino acids from completion; therefore release from the ribosome is likely to occur before significant cytosolic exposure of the nascent chain occurs. This finding coupled with the fact that SRP mediated stalling in bacteria is absent [19] implies that there is only a small time window for targeting events to occur, which therefore favours a pathway of post-translational insertion. In this study we show that F_0c is stable, in the soluble state, and capable of post-translational insertion via YidC and lipid mediated

Abbreviations: DAG, 1-2-dioleoyl-*sn*-glycerol; DDM, n-dodecyl-β-maltoside; F_0c, subunit c of the F_0F_1 ATP synthase; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, lysogeny broth; PK, proteinase K; RNC, ribosome nascent chain; SRP, signal recognition particle; TBS, tris buffered saline

* Corresponding author. Tel.: +44 141 330 5161; fax: +44 141 330 3779.
E-mail address: cheryl.woolhead@glasgow.ac.uk (C.A. Woolhead).

0167-4889/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamcr.2013.07.003

mechanisms. These data presented here provide evidence that YidC can function as a post-translational insertase, a finding that correlates with the absence of a ribosome binding domain, which is present in other members of the YidC family.

2. Materials and methods

2.1. Molecular graphics

All protein structures were drawn using UCSF Chimera package from the Resource for Biocomputing, Visualization and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081) [20].

2.2. Plasmid construction

YidC was amplified by PCR using the primers 5′-CCGGAATTCATG GATTCGCAACGCAATCTTTTAGTCATCGC-3′ (YcEco5) and 5′-CCCAAGCT TTCAGGATTTTTTCTTCTCGCGGCTATG-3′ (YcHind3) and *Escherichia coli* strain MC4100 genomic DNA as a template. The PCR product was digested with EcoRI and HindIII and cloned into pTrc99a (Pharmacia) to create pTrc-YidC. A hexahistidine tag was attached to YidC by first amplifying YidC using the primers YcEco5 and 5′-ACCTCTAGAACCGGATTTTTTCTTCTCGCGGCTATGC-3′ and pTrc-YidC as a template, digesting the PCR product with EcoRI and XbaI, and then recloning it into pTRC99a to create pTrc-YidC (Xba). The oligonucleotides 5′-CTAGAGGTCATCACCATCACCATCACGGCTGAA-3′ and 5′-AGCTTTCAGCCGTGATGGTGATGGTGATGACCT-3′ were then ligated into pTrcYidC (Xba) digested with XbaI and HindIII to add the His tag and create pTrc-YidC(HIS).

For transcription/translation experiments pTrc99a atpE and pF_0c T7 were utilised [18]. For fluorescence experiments purification of F_0c was required and therefore a hexaHistag was added to the C terminus by amplification from pTrc99a atpE using the oligonucleotides 5′-CATGG TACCGGCAGGAAACAGACCATGGAAAACCTGAATATGG-3′ and 5′-GTATC TAGAGTATTATTAGTGATGGTGATGGTGATGCGCGACAGCGAACATCACG-3′; the PCR product was cloned into the KpnI and XbaI sites of pTrc99a to give pTrc99a atpE-His. Site directed mutagenesis to produce the L45C mutant of F_0c was carried out using the QuikChange system (Stratagene).

2.3. Liposome preparation

E. coli polar lipid extract and 1-2-dioleoyl-*sn*-glycerol (DAG) were purchased from Avanti Polar Lipids and stored in chloroform at −20 °C. The desired amount of lipid was dried under a stream of nitrogen to produce a lipid film, which was then left under vacuum overnight to remove trace amounts of chloroform. The lipid films were rehydrated in 20 mM Hepes pH 7.4 and subjected to 5 cycles of freeze–thawing on dry ice. The multilamellar suspension was then extruded through 100 nm pore membranes (Whatman) to form unilamellar liposomes.

2.4. Overexpression, purification and on column reconstitution of His-tagged YidC

E. coli C41 cells were transformed with the plasmid pTrc-YidC(His); 5 ml overnight cultures were grown and used to inoculate 1 l cultures (LB and 0.1 mg/ml ampicillin). Expression was induced through the addition of 1 mM IPTG at an OD of 0.4–0.6, and cells were harvested 2 hours post induction through centrifugation at 3200 *g* for 20 min, 4 °C. Cell pellets were re-suspended in TBS containing lysozyme (1 mg/ml) and the cells were lysed using a French press before centrifugation at 40,000 *g* for 50 min at 4 °C to remove unlysed cells and inclusion bodies. The supernatant was then subjected to ultracentrifugation at 160,000 *g* for 50 min at 4 °C to isolate membrane pellets, which were stored at −80 °C.

Membrane pellets were solubilised in TBS containing 1% DDM (Anatrace), 10 mM β-mercaptoethanol and 40 mM imidazole and applied to a 1 ml His-Gravitrap nickel affinity column (GE Healthcare). The bound protein was washed with 20 column volumes of wash buffer (40 mM Tris pH 8, 300 mM NaCl, 0.03% DDM, 10 mM β-mercaptoethanol), containing 40 mM imidazole and 3 column volumes of wash buffer containing 100 mM imidazole. The column was then equilibrated with detergent saturated liposomes (0.5 mg/ml liposomes, 0.3 mg/ml DDM, 20 mM Hepes pH 7.4) and incubated for 2 hours. Liposomes (0.5 mg/ml, 20 mM Hepes pH 7.4) were then gradually added to the column over a 2 hour period (2 column volumes in total) to gradually replace the detergent with lipid. The column was then washed rapidly with 3 column volumes of 0.1 mg/ml liposomes before adding 3 ml of liposome elution buffer (0.1 mg/ml liposomes, 400 mM Imidazole, 40 mM Tris pH 8, 300 mM NaCl). Purity was assessed through SDS–PAGE. The reconstituted protein was dialysed against 20 mM Hepes pH 7.4 overnight and centrifuged at 20,000 *g* for 10 min before storage at 4 °C. The protein was studied within 2–3 weeks of purification and centrifuged (20,000 *g*) before each experiment to ensure solubility. Orientation was tested by adding trypsin (55 μg/ml) in the absence and presence of 1% triton X-100 before incubation at 4 °C for 2 hours. The reactions were then pelleted by TCA precipitation and washed with acetone before analysis through SDS–PAGE.

2.5. Translation and isolation of soluble F_0c

Linear DNA was amplified from the pTrc99a atpE plasmid using an Ex Taq PCR kit (TaKaRa). In these reactions the 5′ primer was located upstream of the *trc* promoter and the reverse primer was downstream of the stop codon. Purified PCR product was then used to programme coupled transcription/translation reactions to produce ^{35}S-Methionine labelled F_0c. These reactions were performed in varying volumes principally as described previously [18]. The reactions were stopped by adding 20 mM EDTA and the translation product was buffer exchanged into 20 mM Hepes pH 7.4, using centrifugal concentrators (Millipore). The translation product was then processed through 3 × 20 min rounds of ultracentrifugation (434,500 *g*, 4 °C) to remove endogenous membranes and aggregated F_0c. The pellet and supernatant were analysed for the presence of F_0c through tricine SDS–PAGE autoradiography and the same samples were probed for YidC (a marker for endogenous membranes) through western blotting. Digestion of soluble F_0c was analysed by adding PK (0.2 mg/ml) at 4 °C for 2 hours.

Translations using the pure system (NEB) were performed according to manufactures instructions using the plasmid pF_0c T7 [18] to produce linear DNA. The translation product was then processed and analysed as described above.

2.6. Insertion assays

Soluble F_0c produced through the *E. coli* S30 extract system and processed as described above (buffer exchanged and subject to three rounds of ultracentrifugation) were added to reactions (100 μl) containing either empty liposomes or YidC proteoliposomes (0.15 mg/ml YidC concentration) in 20 mM Hepes pH 7.4 with lipid concentrations between 0 and 2 mg/ml. The reactions were incubated at room temperature for 30 min before ultracentrifugation for 30 min at 434,500 *g*, 4 °C to pellet liposomes/proteoliposomes. The pellets were re-suspended in sample buffer and the protein in the supernatant was acetone precipitated. The samples were analysed through tricine SDS–PAGE and autoradiography. For digestion assays the pellets were washed with 3 M potassium acetate and repelleted before resuspension in 20 mM Hepes pH 7.4. PK was added at a concentration of 0.2 mg/ml in the presence or absence of 1% triton X-100.

2.7. Blue Native PAGE

For soluble samples, Blue Native PAGE sample buffer (50 mM Bis-Tris, 50 mM NaCl, 10% glycerol, 0.05% DDM, 0.05% ponceau) was added directly. For membrane samples, YidC-proteoliposomes/empty liposomes containing F_0c produced through the insertion assay were pelleted through ultracentrifugation for 30 min at 434,500 *g*, 4 °C and re-suspended in Blue Native PAGE sample buffer. Samples were loaded directly onto a Native PAGE Novex 4–16% Bis-Tris gel and electrophoresis was performed at 4 °C, using cathode buffer (50 mM Bis-Tris, 50 mM Tricine, 0.02% Coomassie Blue G-250, pH 6.8) and anode buffer (50 mM Bis-Tris HCl, pH 6.8). Gels were fixed in 40% methanol, 10% acetic acid and left in 8% acetic acid overnight. Gels were dried and visualised through autoradiography.

2.8. Calcein release

Liposomes were produced as described in Section 2.3 except that 60 mM calcein (Molecular Probes) was added to the buffer before hydration of the lipid film. Following extrusion, calcein loaded vesicles were separated from free calcein on gel filtration columns (0.7 cm × 50 cm) containing CL-6B Sepharose equilibrated with 20 mM Hepes pH 7.4. To a 375 μl aliquot of vesicles 25 μl of either soluble F_0c (as produced in Section 2.5) or triton (final concentration 0.625%) was added. Fluorescence was measured in a 4 mm path length quartz cuvette (Starna) at 20 °C with a Fluoromax-3 spectrophotometer (Horiba Jobin Yvon) at an excitation wavelength of 485 nm and emission recorded between 500 nm and 650 nm (2 averages), with a bandpass of 1 nm and a 1 s integration time.

2.9. Purification of His tagged F_0c and fluorescence labelling

His tagged F_0c constructs (either wildtype or L45C mutant) were expressed from the pTrc99a atpE-His plasmid in C41 *E. coli* cells and purified as described for YidC up to the point of reconstitution; at this stage the bound protein was instead washed with 3 column volumes of 20 mM Hepes pH 7.4, 0.03% DDM followed by 2 column volumes of the same buffer containing 0.1 mM BODIPY®FL C_1-IA (Molecular Probes). The labelling proceeded for 2 hours before the reaction was quenched with the addition of 5 column volumes of wash buffer (40 mM Tris pH 8, 40 mM Imidazole, 300 mM NaCl, 0.03% DDM) containing 10 mM β-mercaptoethanol. The protein was then washed with a further 5 column volumes of wash buffer without β-mercaptoethanol before labelled F_0c-His was eluted in 2 column volumes of elution buffer (40 mM Tris pH 8, 400 mM Imidazole, 300 mM NaCl, 0.03% DDM). F_0c-His was detected through Tricine SDS–PAGE and Coomassie staining and the identity of the protein confirmed through western blotting using a rabbit anti-his-tag polyclonal antibody (Abcam).

2.10. Insertion of BODIPY labelled F_0c and fluorescence

A 20 μl aliquot of labelled F_0c-His L45C (~1 mg/ml) in 0.03% DDM was added to 400 μl of YidC proteoliposomes (0.08 mg/ml protein concentration) or empty liposomes (2 mg/ml), the reaction was incubated for 30 min at room temperature and then the sample was concentrated to 100 μl and loaded onto a gel filtration column (0.7 cm × 50 cm) containing CL-6B Sepharose equilibrated with 20 mM Hepes pH 7.4. Fractions were collected and analysed for the presence of proteoliposomes/liposomes through tryptophan fluorescence and light scattering. Fluorescence was measured as in Section 2.8 but with a bandpass of 4 nm. The resulting spectra were background corrected against samples containing wildtype F_0c-His that were labelled and purified through the same procedure. Quenching was assessed by adding anti BODIPY®FL rabbit IgG antibody (Molecular Probes) to a concentration of 0.03 mg/ml and measuring the change in fluorescence.

3. Results

3.1. A one step method for the purification and reconstitution of YidC

To study insertion that is exclusively YidC mediated requires purification and reconstitution of YidC into liposomes. We therefore over expressed C-terminally His-tagged YidC in *E. coli* for purification through nickel affinity chromatography. Previous purification methods for His-tagged YidC required downstream purification steps to obtain protein of sufficient purity [7,21,22]. To optimise the purification we added 40 mM imidazole to the load to prevent non-specific binding to the nickel column and added an additional 100 mM imidazole wash step following the standard 40 mM imidazole wash step. These improvements enhanced the purity of the protein on elution and removed any need for downstream purification (Fig. 1A).

Reconstitution of detergent solubilised membrane proteins can be achieved by replacing the detergent with detergent-saturated liposomes, and then gradually removing the detergent until proteoliposomes form. Detergent removal can be achieved through processes including dialysis, dilution and incubation with Bio-beads [23]. To streamline the reconstitution of YidC we coupled the process to the purification by exchanging detergent for detergent saturated liposomes whilst the pure protein is still bound to the column. The detergent saturated liposomes were then exchanged gradually for intact liposomes, diluting the detergent away

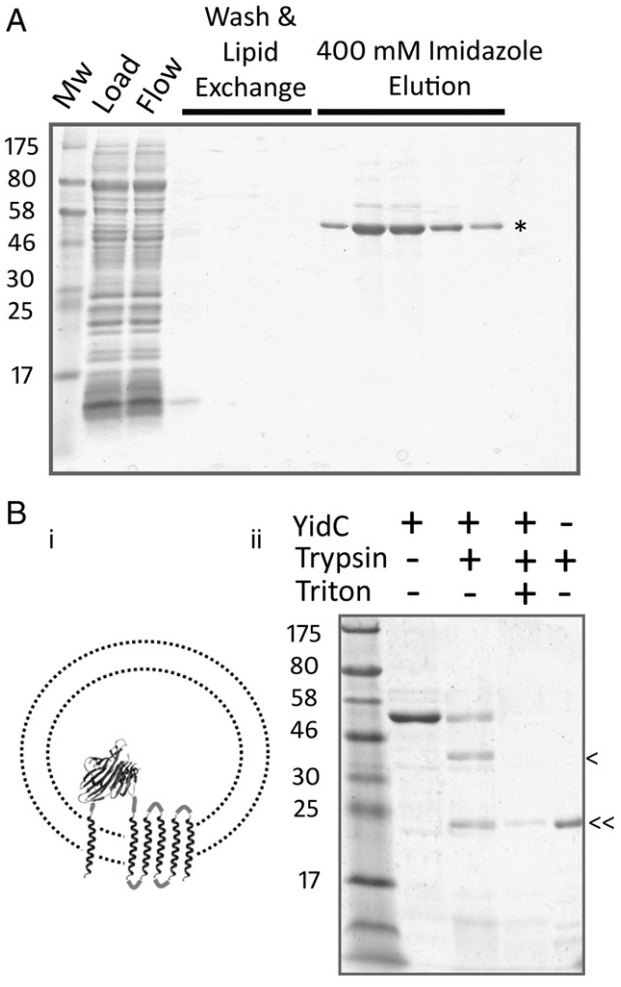

Fig. 1. Purification and membrane reconstitution of YidC. SDS–PAGE showing (A) purification progression of His-tagged YidC (*). (B i) Diagram illustrating protection of the periplasmic domain when YidC is correctly orientated. The periplasmic domain was drawn using PDB file 3BS6. (B ii) SDS–PAGE of trypsin digestion to assess membrane orientation of purified YidC. Arrow highlights the periplasmic fragment and the double arrow highlights trypsin. Molecular weight markers are in kDa.

until purified protein incorporates into the liposomes before elution. This enabled purified reconstituted YidC to be obtained from a detergent solubilised membrane pellet, within a single day. Furthermore, exchanging lipid for detergent on the column ensures that the His-Tag remains on the outer face of the liposome as the bilayer forms around the protein that enhances the population of YidC in the correct membrane orientation, with the periplasmic domain internalised (Fig. 1Bi). Tryptic digestion was used to assess protection of the periplasmic domain (Fig. 1Bii). Digestion results in a characteristic band on a Coomassie stained SDS–PAGE, which was identified as the periplasmic domain through mass spectrometry. There is still some undigested YidC present; this could be due to the reduced accessibility of trypsin for membrane embedded YidC, which lowers digestion efficiency. Although affinity columns have previously been used for detergent exchange [23], this is to our knowledge the first time that such a method has been used for reconstitution. This has applications for the rapid purification and reconstitution of membrane proteins from a variety of lipid systems, especially when a specific protein orientation is desired.

3.2. F_0c is soluble in the absence of a hydrophobic support and oligomerises in the soluble state

To investigate membrane insertion we chose F_0c as a model YidC dependent substrate. F_0c is a 79 amino acid protein that forms a helical hairpin in the membrane (Fig. 2Ai) and oligomerises into a decamer (Fig. 2Aii) to form a proton conducting channel. From our earlier studies on co-translational folding, the signal anchor domain of F_0c was shown to compact inside the ribosomal exit tunnel and to begin association with SRP when synthesis was close to completion [18]. This led us to hypothesise that release from the ribosome could occur before completion of targeting, thus favouring a post-translational mechanism of membrane insertion. To test this hypothesis we developed an experimental system to couple full length released F_0c to YidC mediated membrane insertion. We produced full length F_0c through *in vitro* translation using an *E. coli* cell extract programmed with PCR product and detected translation through radiolabelling and Tricine SDS–PAGE (Fig. 2Bi).

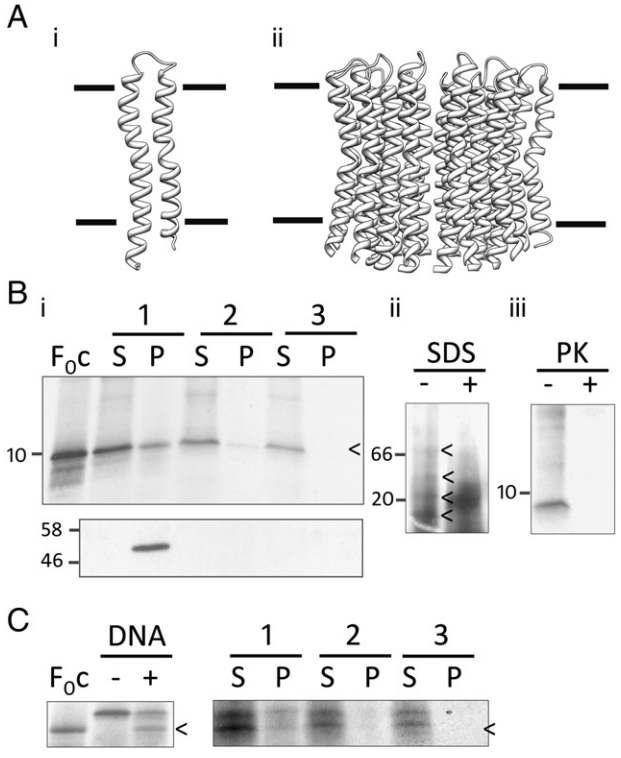

Fig. 2. Soluble F_0c is PK sensitive and forms a mixture of oligomers. (A) NMR structures of F_0c in the (i) monomeric and (ii) native decamer state, drawn using PDB files 1C0V and 1C17. (B i) Autoradiograph detecting the presence of F_0c produced through *in vitro* translation in the supernatant (S) and pellet (P) following each of 3 rounds (1,2,3) of ultracentrifugation. Western blot analysis (lower panel) of fractionated samples probed with anti-YidC antisera. (ii) Blue Native PAGE analysis of soluble F_0c in the absence (−) and presence (+) of SDS, arrows indicate multiple species present. (iii) PK digestion of soluble F_0c (C) Autoradiograph detecting the expression and solubility of F_0c using the pure translation system. Reactions were performed with (+) and without (−) DNA template and compared to that produced through S30 translations (F_0c). Solubility was assessed by comparing the supernatant (S) and pellet (P) following each of three rounds (1,2,3) of ultracentrifugation. Molecular weight markers are in kDa.

On incubation of the translation product with liposomes/proteoliposomes, the reactions extensively aggregated. Our analysis indicates that this was due to the presence of magnesium ions, which are essential for retaining the ribosome structure but are known to aggregate liposomes [24]. Therefore it was necessary to buffer exchange the translation product on completion of translation. Furthermore, as the *E. coli* S30 extract contains endogenous membranes, it is preferable to remove these before adding liposomes. The buffer exchanged translation product was therefore subjected to ultracentrifugation at 434,500 *g* to remove any aggregates and lipid membranes present. At this stage we were unsure if F_0c would remain soluble or co-sediment with the lipids; therefore we analysed both the supernatant and the pellets for the presence of F_0c. Following the first round of ultracentrifugation F_0c was found in both the pellet and the supernatant (Fig. 2Bi); therefore although some F_0c is insoluble and associates with membranes, some surprisingly remains in the soluble phase. When subjecting the supernatant to two further rounds of ultracentrifugation F_0c remained in the supernatant and was purified away from insoluble F_0c. Endogenous YidC was used as a marker for the inner membrane to confirm removal of lipid and was detected through western blotting. YidC was detected in the pellet following the first round of centrifugation and was absent in the other fractions; therefore the membranes in the S30 extract are efficiently separated from F_0c that remains in the soluble state.

When this soluble fraction was analysed through Blue Native PAGE multiple species were revealed that migrate at higher molecular weight values to that expected for F_0c (Fig. 2Bii). This shows that F_0c is either oligomerising or binding to other proteins. When SDS is added to the sample a lower molecular weight smear is detected suggesting that the oligomers dissociate into SDS-bound monomers or stabilising cofactors dissociate. Although ultracentrifugation should be sufficient for lipid removal, residual lipid could be associating with F_0c. Soluble F_0c was susceptible to PK digestion (Fig. 2Biii), which shows that the protein is exposed and unprotected. However to prove solubility in the absence of lipid ideally requires a reconstituted translation system that does not contain lipid. We therefore expressed F_0c using the Pure translation system (NEB), which is made up of purified components that are essential for translation, but lack lipid membranes and accessory factors. Expression of F_0c in this system was lower, which is common for proteins expressed through the Pure system [25]. As shown in Fig. 2C, F_0c remains in the soluble state following each of three rounds of centrifugation. This not only confirms that F_0c remains soluble in the absence of lipid but the lack of chaperones or accessory factors present in this pure translation system suggests that the solubility is an intrinsic property of the protein.

3.3. Post-translational insertion of F_0c

Following the isolation of soluble F_0c, produced using the S30 translation system, the next step was to investigate whether the protein is insertion competent. The soluble fraction that contains F_0c will also contain any soluble proteins present in the *E. coli* S30 extract; this includes targeting factors and chaperones that are required for insertion or maintaining F_0c solubility. The soluble fraction can then be combined with empty liposomes or YidC proteoliposomes and insertion assessed through pelleting under ultracentrifugation. Membrane pellets were

washed with 3 M potassium acetate to remove any peripherally associated protein; the pellets were then treated with PK and analysed through tricine SDS–PAGE and autoradiography for the presence of F_0c (Fig. 3A).

On incubation with empty liposomes (0.1 mg/ml) F_0c remained in the soluble phase and did not insert (Fig. 3Ai). When incubated with YidC-proteoliposomes, at the same lipid concentration, F_0c co-sediments and remains in the pellet after washing with 3 M potassium acetate. Following PK digestion F_0c is still present, which shows a degree of proteinase protection. Furthermore, there is still some protection when Triton is added, which suggests an association with YidC that protects F_0c from PK. The results show that purified reconstituted YidC is functioning as an active insertase and is capable of mediating insertion of F_0c. The insertion detected is post-translational as three steps in the preparation ensure this: (i) the translation product is full length and therefore released from the ribosome before insertion begins, (ii) translation is stopped by the addition of EDTA and subsequent buffer exchange to denature and inactivate the ribosomes, and (iii) ultracentrifugation will remove any ribosomes that are still active following this treatment. These steps all occur before the lipid vesicles are added.

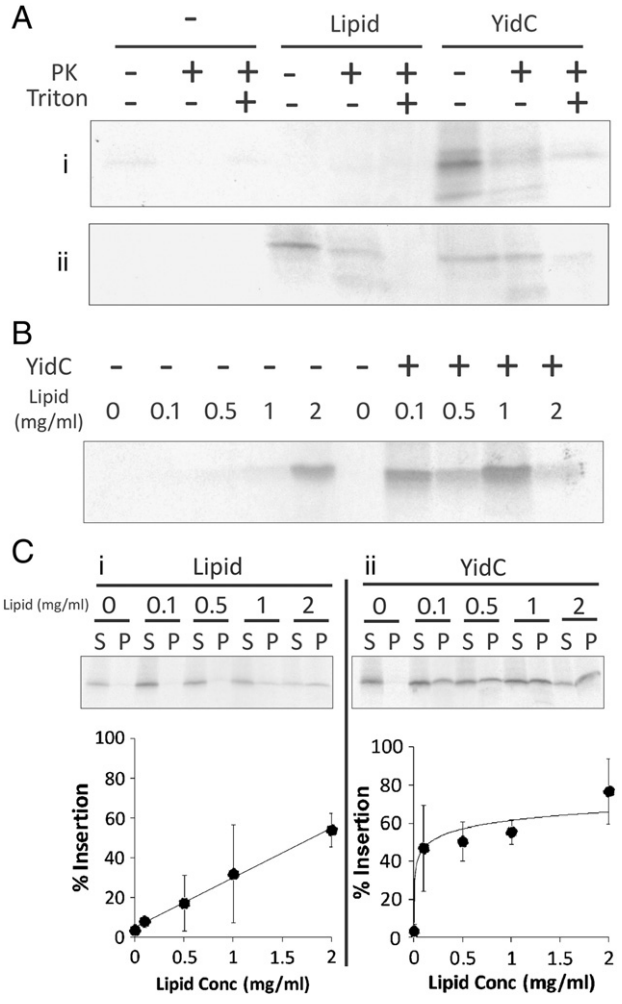

Fig. 3. YidC dependent and independent post-translational insertion (A) PK digestion of resuspended pellets in the absence (−) and presence (Lipid) of liposomes and in the presence of YidC proteoliposomes (YidC). Assays were performed at a lipid concentration of (i) 0.1 mg/ml and (ii) 2 mg/ml. (B) Sedimented membrane pellets analysed for the presence of F_0c at a range of lipid concentrations in the absence and presence of YidC. (C) Quantification of (i) lipid mediated insertion and (ii) YidC mediated insertion. Levels of F_0c in the supernatant (S) and pellet (P) were quantified and plotted. Error bars represent the standard deviation of three independent experiments; fits are for guidance only.

On increasing the lipid concentration to 2 mg/ml insertion occurs with empty liposomes and proteoliposomes that contain YidC (Fig. 3Aii); therefore spontaneous membrane insertion is evident when the lipid concentration is raised to this level. To investigate further the lipid dependence of insertion, assays were performed across a range of lipid concentrations (Fig. 3B and C). In the presence of YidC, co-sedimentation occurred at all lipid concentrations tested at average values between ~40 and 80%. In the absence of YidC insertion was low until a lipid concentration of 1–2 mg/ml was reached at which point insertion levels of up to ~50% were detected. This reveals two types of post-translational F_0c insertion in this experimental system. One that is lipid dependent and occurs in the absence of an active insertase, the other which is YidC facilitated. The concentration dependence of spontaneous insertion indicates an equilibrium process that is pushed in favour of insertion, when the number of liposomes is increased relative to the amount of F_0c. Despite F_0c solubility, it is a very hydrophobic protein and this hydrophobicity will be protected in the lipid bilayer environment. This increased stability in the lipid phase is likely to drive spontaneous insertion as the lipid concentration increases.

3.4. Spontaneous insertion occurs in the presence of diacyl glycerol and does not disrupt the membrane

Diacyl glycerol (DAG) has been reported to prevent spontaneous insertion by filling open spaces in the lipid bilayer and therefore sealing the membrane [26]. However introduction of DAG to the *E. coli* lipid extract did not affect spontaneous insertion in this system (Fig. 4A). To ensure that the lipid bilayer retains its integrity we tested the stability of the vesicles using a calcein release assay. Calcein retained in liposomes at high concentrations self quenches; however if the membrane leaks and the calcein is released it dilutes and therefore dequenches [27]. Stability can therefore be assessed by measuring the change in fluorescence of calcein loaded vesicles (with or without DAG) when they are disrupted with triton. We found that for both types of vesicles fluorescence was much lower before the addition of triton (Fig. 4Bi and ii), which shows that under native conditions the vesicles are stable and retain their contents. However vesicles containing DAG show a ~5 fold increase in fluorescence on triton disruption as opposed to a ~2 fold increase without DAG. This shows that DAG lowers the permeability of the vesicles before the addition of triton to produce a greater change in fluorescence once disrupted. Upon addition of the processed translation mixture (containing soluble F_0c) to fresh vesicles there was no detectable change in fluorescence (Fig. 4Bi and ii) for either lipid system, which shows that the vesicles retain their integrity throughout insertion. This indicates that the spontaneous insertion detected here is not a result of membrane leakage.

3.5. YidC is required for F_0c to adopt a native oligomerisation state but the correct membrane orientation is achieved independently

To analyse the oligomerisation state of F_0c when inserting through YidC dependent and independent mechanisms, membrane pellets were re-suspended in native running buffer and analysed through Blue Native PAGE (Fig. 4C). When insertion is solely dependent on YidC (at a lipid concentration of 0.1 mg/ml) a single prominent band at ~70 kDa is present; this indicates a single oligomerised species that corresponds to a decamer. Native F_0c exists as a decamer as part of the F_0F_1 ATP synthase complex [28]; it therefore appears that post-translational YidC mediated insertion results in this native structure. At a lipid concentration of 2 mg/ml when spontaneous insertion is detected, a range of oligomersied species were visible. This indicates that although F_0c can insert into membranes without an insertase, YidC is required for the protein to adopt its native quaternary structure. This gives evidence for the chaperone function of YidC.

To assess if F_0c maintains a native orientation following insertion, a cysteine residue was introduced into the loop region at position 45

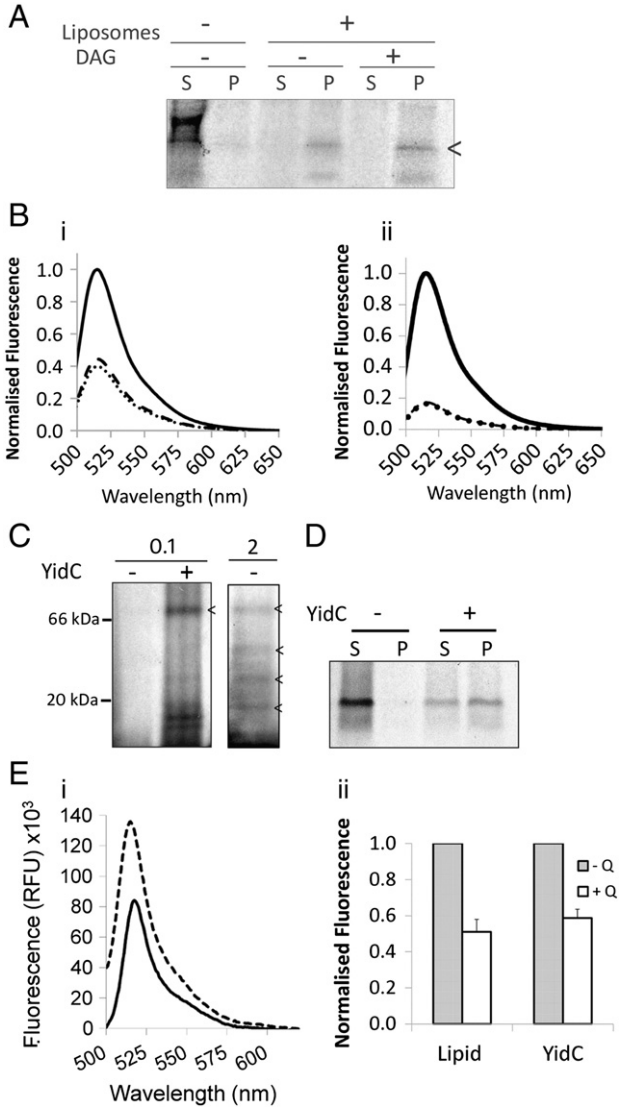

Fig. 4. Conformational characterisation of inserted F_0c and DAG influence on membrane integrity and spontaneous insertion. (A) Sedimentation assay to test if DAG inhibits insertion into liposomes. Sedimented membrane pellets (P) were analysed for the presence of F_0c and compared to supernatant samples (S). (B) Calcein release from (i) liposomes or (ii) DAG containing liposomes before (dotted) and after (dashed) addition of soluble F_0c and after the addition of triton (bold). (C) Blue Native PAGE analysis of inserted F_0c in the presence of YidC (0.1 mg/ml lipid concentration) and following spontaneous insertion into empty liposomes (2 mg/ml). (D) His-tagged F_0c-L45C insertion into YidC proteoliposomes assessed through sedimentation and analysis of the supernatant (S) and pellet (P) for the presence of His-tagged F_0c-L45C. (E) (i) Fluorescence spectra of His-tagged F_0c-L45C labelled with BODIPY FL following insertion into proteoliposomes treated with (solid) or without (dashed) quencher. (ii) Average change in fluorescence on addition of quencher (Q) following insertion into liposomes (lipid) or YidC proteoliposomes (YidC). The error bars show the standard deviation from 3 independent experiments.

and a His tag at the C-terminus was added for purification. F_0c remained insertion competent despite these modifications (Fig. 4D). F_0c L45C was labelled and purified on a nickel column in DDM, concentrated and then added to YidC proteoliposomes or empty liposomes. The proteoliposomes or liposomes containing the inserted protein were then isolated on a gel filtration column and fluorescence was measured before and after addition of quencher (Fig. 4Ei). If F_0c inserts in the correct orientation then the loop region should be solvent exposed. DDM solubilised F_0c L45C (in which the loop region should be fully accessible) showed quenching which saturated at 46% of the original signal; therefore if F_0c inserts in the correct orientation, quenching would be expected around this level. Quenching was detected at a level of ~59% for YidC inserted F_0c and ~51% for lipid inserted F_0c (Fig. 4Eii), which shows a level of accessibility comparable to DDM solubilised F_0c. This indicates that F_0c inserts into both liposomes and YidC proteoliposomes in the same native membrane orientation, with the loop region outside the vesicle. Coupling this finding with the data from the Blue Native PAGE experiments suggests that YidC is required for F_0c to form its correct oligomerisation state but the correct membrane orientation is achieved independently.

4. Discussion and conclusions

Despite the essential function of YidC its mechanistic details are difficult to define. In this study we developed an experimental system, which uncouples translation and membrane insertion, to show that YidC can insert the endogenous substrate F_0c through a post-translational mechanism.

4.1. Post-translational YidC mediated insertion

Previous attempts to reconstitute YidC mediated insertion of F_0c have titrated inner membrane vesicles or proteoliposomes directly into translation reactions. Insertion was detected in these systems but the evidence to resolve whether it is a post or co-translational process is limited. Previously, translation was uncoupled from insertion through the addition of chloramphenicol before the addition of proteoliposomes [14]. This experiment showed minimal insertion, which led the authors to conclude that in the absence of post-translational insertion F_0c insertion occurs co-translationally. In our experimental approach we buffer exchange the translation product and separate F_0c from aggregates and membranes before the addition of proteoliposomes. These processes (i) enrich the sample for soluble, insertion competent F_0c and (ii) provide an environment in which YidC proteoliposomes are stable and free from aggregation. These changes could explain the increased levels of post-translational insertion detected. Our results therefore show the first clear demonstration that F_0c translation can be uncoupled from YidC mediated insertion. This finding does not exclude the possibility that co-translational insertion still takes place *in vivo*; however numerous experimental findings support our hypothesis of a post-translational insertion mechanism, for example, changes to the charge distribution in the loop region of F_0c influence insertion [29]. This loop region is believed to interact with YidC; although it must be protected by the ribosome tunnel until termination and release, this could be explained by the use of a post-translational insertion mechanism. Furthermore, our previous work showed that co-translational compaction of F_0c delays exposure to the cytoplasm until translation is close to completion [18]; this and the absence of SRP mediated stalling in bacteria favours ribosome release before successful targeting. Finally in mitochondria there is evidence for post-translational insertion of the F_0c homologue atp9 [30], which utilises the YidC homologue Oxa1 for insertion. Therefore there is evidence of post-translational insertion in another YidC family member.

If our assumptions are correct and the late exposure of a signal anchor sequence, due to the short length of F_0c, is a specific characteristic that favours post-translational targeting and insertion then this has implications for the targeting of other YidC substrates. Many of which are short and therefore have a small time window for targeting [1]. Although this has not been specifically investigated, there are indications that the exogenous YidC substrate Pf3 inserts through a post-translational mechanism as direct substrate interactions have been reported [13,31]. Furthermore, a recently identified YidC dependant substrate is a tail anchored membrane protein [32], which must insert through a post-translational mechanism due to the ribosomal protection of its C-terminal signal anchor sequences up until release from the ribosome. Numerous YidC substrates therefore contain signal anchor sequences that are only exposed near the end of translation, a property which promotes a post-translational mechanism of insertion.

The role of SRP in YidC targeting has been difficult to define, with different experimental systems yielding different conclusions [14,18,33–35]. How does the finding that YidC has post-translational insertion activity fit with the mechanisms of SRP mediated targeting? When insertion is post-translational, the targeting mechanisms are likely to be SRP independent, as SRP dissociates from signal anchor sequences upon ribosome release [36]. Our results presented here and in our previous study [18] favour a model in which SRP promotes but is not essential for YidC facilitated insertion. We propose that SRP may improve the efficiency of F_0c targeting by positioning translating ribosomes at the membrane, which increases the proximity of the substrate to YidC (Fig. 5) and improves its diffusion rate on release to aid direct delivery. This interpretation would explain why SRP depletion lowers insertion efficiency *in vivo* whilst also consistent with the non-essential role of SRP when translation/insertion systems are reconstituted *in vitro*.

4.2. A post-translational insertion mechanism explains the absence of a ribosome binding domain

It is interesting to consider how the post-translational activity of YidC correlates with other members of the same protein family. Gram-negative bacteria contain one member of the YidC family but there are 2 homologues in mitochondria (Cox18 and Oxa1), chloroplasts (Alb3 and Alb4) and gram positive bacteria (YidC1 and YidC2) [37]. A notable feature of Oxa1, Alb4 and YidC2 is an extended C-terminus that functions as a ribosome binding domain, which is absent in other YidC family members. Of the YidC family members that lack the extended C-terminus Cox18 and Alb3 have been shown to have post-translational activity [38,39]. The lack of a C-terminal ribosome binding domain on YidC and the fact that the entire C-terminus is non-essential for activity [40] indicate that either YidC mediated insertion is independent of ribosome binding, or an alternative site recognises the ribosome. Here we have shown that YidC can insert F_0c following the completion of translation and in the absence of active ribosomes. This shows that YidC is capable of direct recognition of released substrates and therefore suggests that YidC mediated insertion does not require direct interaction with the ribosome. This finding brings YidC activity into alignment with other members of the family that show post-translational activity in the absence of an extended, ribosome binding, C-terminus.

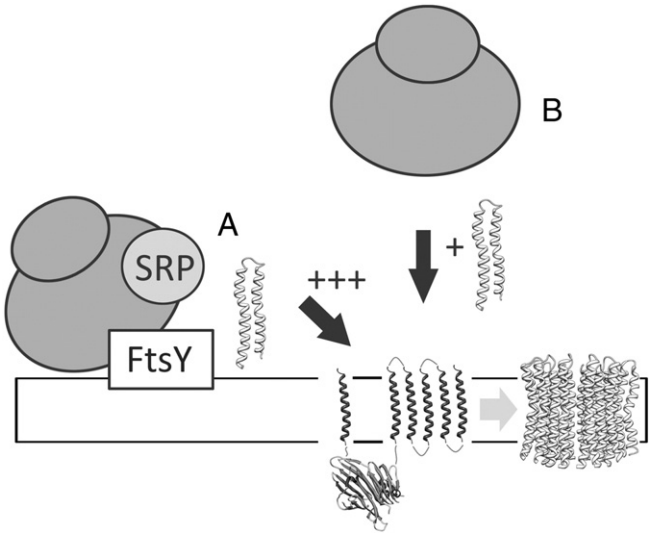

Fig. 5. Model for the post-translational targeting of F_0c. (A) Ribosomes targeted to the SRP receptor (FtsY) release F_0c, which then interacts directly with YidC, resulting in efficient insertion (+++). (B) F_0c that is released before targeting diffuses to the membrane, interacts with YidC and inserts. The lack of targeting lowers the efficiency (+) but not integrity of insertion.

4.3. Wider role of YidC as a post-translational insertase

Our results support a model in which YidC directly recognises and inserts released protein substrates, rather than ribosome bound intermediates. This model points towards a function of YidC in the insertion of proteins whose early release prevents efficient targeting to the Sec dependent pathway. Extrapolating this ability to recognise and insert released membrane proteins may point to a broader function of YidC, to rescue insertion of prematurely released membrane proteins and provide a backup system to the more tightly controlled Sec system.

Acknowledgements

The authors wish to thank Dr. Robert Boulianne for assistance in the construction of the pTrc-YidC(HIS) plasmid, Dr. Harris Bernstein for providing the YidC antibody and Jane Findlay for construction of the His-F_0c plasmid. This project was funded by Biotechnology and Biological Sciences Research Council Grant [BB/G011281].

References

[1] R.E. Dalbey, P. Wang, A. Kuhn, Assembly of bacterial inner membrane proteins, Annu. Rev. Biochem. 80 (2011) 161–187.
[2] D.J. du Plessis, N. Nouwen, A.J. Driessen, The Sec translocase, Biochim. Biophys. Acta 1808 (2011) 851–865.
[3] G. von Heijne, Sec-independent protein insertion into the inner *E. coli* membrane. A phenomenon in search of an explanation, FEBS Lett. 346 (1994) 69–72.
[4] S.J. Facey, A. Kuhn, Biogenesis of bacterial inner-membrane proteins, Cell. Mol. Life Sci. 67 (2010) 2343–2362.
[5] A.N. Gray, J.M. Henderson-Frost, D. Boyd, S. Sharafi, H. Niki, M.B. Goldberg, Unbalanced charge distribution as a determinant for dependence of a subset of *Escherichia coli* membrane proteins on the membrane insertase YidC, MBio. 2 (2011).
[6] D. Kiefer, A. Kuhn, Hydrophobic forces drive spontaneous membrane insertion of the bacteriophage Pf3 coat protein without topological control, EMBO J. 18 (1999) 6299–6306.
[7] J. Serek, G. Bauer-Manz, G. Struhalla, L. van den Berg, D. Kiefer, R. Dalbey, A. Kuhn, *Escherichia coli* YidC is a membrane insertase for Sec-independent proteins, EMBO J. 23 (2004) 294–301.
[8] J.C. Samuelson, M. Chen, F. Jiang, I. Moller, M. Wiedmann, A. Kuhn, G.J. Phillips, R.E. Dalbey, YidC mediates membrane protein insertion in bacteria, Nature 406 (2000) 637–641.
[9] M.L. Urbanus, L. Froderberg, D. Drew, P. Bjork, J.W. de Gier, J. Brunner, B. Oudega, J. Luirink, Targeting, insertion, and localization of *Escherichia coli* YidC, J. Biol. Chem. 277 (2002) 12718–12723.
[10] A.J. Driessen, How proteins cross the bacterial cytoplasmic membrane, J. Membr. Biol. 142 (1994) 145–159.
[11] S. Nagamori, I.N. Smirnova, H.R. Kaback, Role of YidC in folding of polytopic membrane proteins, J. Cell Biol. 165 (2004) 53–62.
[12] P.A. Scotti, M.L. Urbanus, J. Brunner, J.W. de Gier, G. von Heijne, C. van der Does, A.J. Driessen, B. Oudega, J. Luirink, YidC, the *Escherichia coli* homologue of mitochondrial Oxa1p, is a component of the Sec translocase, EMBO J. 19 (2000) 542–549.
[13] M. Chen, J.C. Samuelson, F. Jiang, M. Muller, A. Kuhn, R.E. Dalbey, Direct interaction of YidC with the Sec-independent Pf3 coat protein during its membrane protein insertion, J. Biol. Chem. 277 (2002) 7670–7675.
[14] M. van der Laan, P. Bechtluft, S. Kol, N. Nouwen, A.J. Driessen, F_1F_0 ATP synthase subunit c is a substrate of the novel YidC pathway for membrane protein biogenesis, J. Cell Biol. 165 (2004) 213–222.
[15] L. Yi, F. Jiang, M. Chen, B. Cain, A. Bolhuis, R.E. Dalbey, YidC is strictly required for membrane insertion of subunits a and c of the F_1F_0ATP synthase and SecE of the SecYEG translocase, Biochemistry 42 (2003) 10537–10544.
[16] M. van der Laan, M.L. Urbanus, C.M. Ten Hagen-Jongman, N. Nouwen, B. Oudega, N. Harms, A.J. Driessen, J. Luirink, A conserved function of YidC in the biogenesis of respiratory chain complexes, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 5801–5806.
[17] S. Kol, N. Nouwen, A.J. Driessen, Mechanisms of YidC-mediated insertion and assembly of multimeric membrane protein complexes, J. Biol. Chem. 283 (2008) 31269–31273.
[18] P.J. Robinson, J.E. Findlay, C.A. Woolhead, Compaction of a prokaryotic signal-anchor transmembrane domain begins within the ribosome tunnel and is stabilized by SRP during targeting, J. Mol. Biol. 423 (2012) 600–612.
[19] T. Powers, P. Walter, Co-translational protein targeting catalyzed by the *Escherichia coli* signal recognition particle and its receptor, EMBO J. 16 (1997) 4880–4886.
[20] E.F. Pettersen, T.D. Goddard, C.C. Huang, G.S. Couch, D.M. Greenblatt, E.C. Meng, T.E. Ferrin, UCSF Chimera—a visualization system for exploratory research and analysis, J. Comput. Chem. 25 (2004) 1605–1612.
[21] D. Martinez Molina, A.K. Lundback, D. Niegowski, S. Eshaghi, Expression and purification of the recombinant membrane protein YidC: a case study for increased stability and solubility, Protein Expr. Purif. 62 (2008) 49–52.

[22] S. Winterfeld, N. Imhof, T. Roos, G. Bar, A. Kuhn, U. Gerken, Substrate-induced conformational change of the *Escherichia coli* membrane insertase YidC, Biochemistry 48 (2009) 6684–6691.
[23] A.M. Seddon, P. Curnow, P.J. Booth, Membrane proteins, lipids and detergents: not just a soap opera, Biochim. Biophys. Acta 1666 (2004) 105–117.
[24] N. Duzgunes, S. Nir, J. Wilschut, J. Bentz, C. Newton, A. Portis, D. Papahadjopoulos, Calcium- and magnesium-induced fusion of mixed phosphatidylserine/phosphatidylcholine vesicles: effect of ion binding, J. Membr. Biol. 59 (1981) 115–125.
[25] J.R. Hillebrecht, S. Chong, A comparative study of protein synthesis in in vitro systems: from the prokaryotic reconstituted to the eukaryotic extract-based, BMC Biotechnol. 8 (2008) 58.
[26] Y. Kawashima, E. Miyazaki, M. Muller, H. Tokuda, K. Nishiyama, Diacylglycerol specifically blocks spontaneous integration of membrane proteins and allows detection of a factor-assisted integration, J. Biol. Chem. 283 (2008) 24489–24496.
[27] T.M. Allen, L.G. Cleland, Serum-induced leakage of liposome contents, Biochim. Biophys. Acta 597 (1980) 418–426.
[28] W. Jiang, J. Hermolin, R.H. Fillingame, The preferred stoichiometry of c subunits in the rotary motor sector of *Escherichia coli* ATP synthase is 10, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 4966–4971.
[29] S. Kol, N. Nouwen, A.J. Driessen, The charge distribution in the cytoplasmic loop of subunit C of the F_1F_0 ATPase is a determinant for YidC targeting, J. Biol. Chem. 283 (2008) 9871–9877.
[30] L. Jia, M.K. Dienhart, R.A. Stuart, Oxa1 directly interacts with Atp9 and mediates its assembly into the mitochondrial F_1Fo-ATP synthase complex, Mol. Biol. Cell 18 (2007) 1897–1908.
[31] S. Ernst, A.K. Schonbauer, G. Bar, M. Borsch, A. Kuhn, YidC-driven membrane insertion of single fluorescent Pf3 coat proteins, J. Mol. Biol. 412 (2011) 165–175.
[32] M.S. Aschtgen, A. Zoued, R. Lloubes, L. Journet, E. Cascales, The C-tail anchored TssL subunit, an essential protein of the enteroaggregative *Escherichia coli* Sci-1 Type VI secretion system, is inserted by YidC, Microbiologyopen 1 (2012) 71–82.
[33] L. Yi, N. Celebi, M. Chen, R.E. Dalbey, Sec/SRP requirements and energetics of membrane insertion of subunits a, b, and c of the Escherichia coli F_1F_0 ATP synthase, J. Biol. Chem. 279 (2004) 39260–39267.
[34] S.J. Facey, S.A. Neugebauer, S. Krauss, A. Kuhn, The mechanosensitive channel protein MscL is targeted by the SRP to the novel YidC membrane insertion pathway of *Escherichia coli*, J. Mol. Biol. 365 (2007) 995–1004.
[35] E. van Bloois, G. Jan Haan, J.W. de Gier, B. Oudega, J. Luirink, F(1)F(0) ATP synthase subunit c is targeted by the SRP to YidC in the *E. coli* inner membrane, FEBS Lett. 576 (2004) 97–100.
[36] J. Luirink, S. High, H. Wood, A. Giner, D. Tollervey, B. Dobberstein, Signal-sequence recognition by an *Escherichia coli* ribonucleoprotein complex, Nature 359 (1992) 741–743.
[37] S. Funes, F. Kauff, E.O. van der Sluis, M. Ott, J.M. Herrmann, Evolution of YidC/Oxa1/Alb3 insertases: three independent gene duplications followed by functional specialization in bacteria, mitochondria and chloroplasts, Biol. Chem. 392 (2011) 13–19.
[38] S. Funes, F.E. Nargang, W. Neupert, J.M. Herrmann, The Oxa2 protein of *Neurospora crassa* plays a critical role in the biogenesis of cytochrome oxidase and defines a ubiquitous subbranch of the Oxa1/YidC/Alb3 protein family, Mol. Biol. Cell 15 (2004) 1853–1861.
[39] M. Moore, M.S. Harrison, E.C. Peterson, R. Henry, Chloroplast Oxa1p homolog albino3 is required for post-translational integration of the light harvesting chlorophyll-binding protein into thylakoid membranes, J. Biol. Chem. 275 (2000) 1529–1532.
[40] F. Jiang, M. Chen, L. Yi, J.W. de Gier, A. Kuhn, R.E. Dalbey, Defining the regions of *Escherichia coli* YidC that contribute to activity, J. Biol. Chem. 278 (2003) 48965–48972.