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.