Protein Expression and Purification 101 (2014) 138–145

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Expression, purification and functional reconstitution of FeoB, the ferrous iron transporter from *Pseudomonas aeruginosa*

Saeed Seyedmohammad [a], Diana Born [a], Henrietta Venter [b,*]

[a] Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD, UK
[b] School of Pharmacy & Medical Sciences, Sansom Institute for Health Research, University of South Australia, GPO Box 2471, Adelaide 5001, Australia

ARTICLE INFO

Article history:
Received 6 March 2014
and in revised form 20 June 2014
Available online 30 June 2014

Keywords:
Pseudomonas aeruginosa
Iron transporter
Membrane protein
Overexpression
Reconstitution

ABSTRACT

The FeoB Fe(II) transporter from the drug resistant pathogen, *Pseudomonas aeruginosa* is essential for ferrous iron transport and is implicated in virulence and biofilm development. Hence it is an attractive target for the development of new anti-infective drugs. FeoB is an intriguing protein that consists of a cytosolic N-terminal GTPase domain and an integral membrane domain which most likely acts as ferrous iron permease. Characterisation of FeoB is critical for developing therapeutics aimed at inhibiting this protein. However, structural and functional analysis of FeoB is hampered by the lack of high yield homogenously pure protein which is monodisperse, stable and active in solution. Here we describe the optimised procedure for the recombinant expression of FeoB from *P. aeruginosa* and provide an evaluation of the most favourable purification, pH and detergent conditions. The functional reconstitution of FeoB in liposomes is also described. This represents the first detailed procedure for obtaining a pure, active and stable FeoB solution in milligram quantities which would be amenable to biochemical, biophysical and structural studies.

Introduction

Pseudomonas aeruginosa is a Gram-negative pathogen which is associated with a range of life-threatening hospital acquired infections. It is also the main cause of mortality in patients suffering from Cystic Fibrosis and is characterised by an innate resistance against multiple classes of antimicrobials [1–4]. Antimicrobial resistance amongst clinical isolates of *P. aeruginosa* is increasing at an alarming rate and has become a major health problem [2,5,6], hence there is an urgent need to discover new treatments and therapies against *P. aeruginosa*. An important way of fighting infection is the targeting of bacterial iron acquisition. Iron is vital for the survival of pathogens as well as being an essential constituent of virulence and biofilm formation [7,8]. Ferrous iron is acquired by the Feo transporter which is composed of three proteins, FeoA, FeoB and FeoC (Fig. 1A and B) [9]. In this transport system FeoB is the iron permease protein, which contains a cytosolic N-terminal domain with GTPase activity and an integral membrane domain which could function as the Fe(II) permease domain. Ferrous iron acquisition through the membrane domain is dependent on the GTPase activity of the soluble domain [10,11]. Deletion of the *feoB* gene has a detrimental effect on the ability of pathogens to form biofilms and also attenuates their virulence [12–20]. FeoB is therefore a tractable target for inhibition. Despite its important role in bacterial survival and virulence, our knowledge about this transporter is still in its infancy. Establishing the structure and molecular mechanism of the FeoB protein would be essential for the development of therapies that target this protein. Although the crystal structures of the N-terminal GTP binding domain of FeoB (NFeoB) proteins from various bacteria have recently been obtained [21–30], this data has yet to be translated into structural and functional significance for the full-length FeoB protein. Due to the difficulties in obtaining high-level overexpression and functional purification of integral membrane proteins, no structures of full-length FeoB from any organisms has been solved yet. Key questions remain regarding the structure of the membrane domain, the coupling between GTP hydrolysis and iron transport, the iron translocation pathway through the FeoB permease domain and the role of FeoA and FeoC in iron transport by FeoB. Progress on FeoB has been hampered by the difficulty in obtaining sufficient yields of homogenously pure, monodisperse, active full-length protein. In this study we have addressed these issues in order to obtain purified FeoB which should be amenable to biochemical, biophysical and structural studies. We have heterologously expressed FeoB from *P. aeruginosa* in *Escherichia coli* and have optimised the conditions of expression and purification. The

* Corresponding author. Tel.: +61 8 83021515; fax: +61 8 83022389.
E-mail address: rietie.venter@unisa.edu.au (H. Venter).

http://dx.doi.org/10.1016/j.pep.2014.06.012
1046-5928/

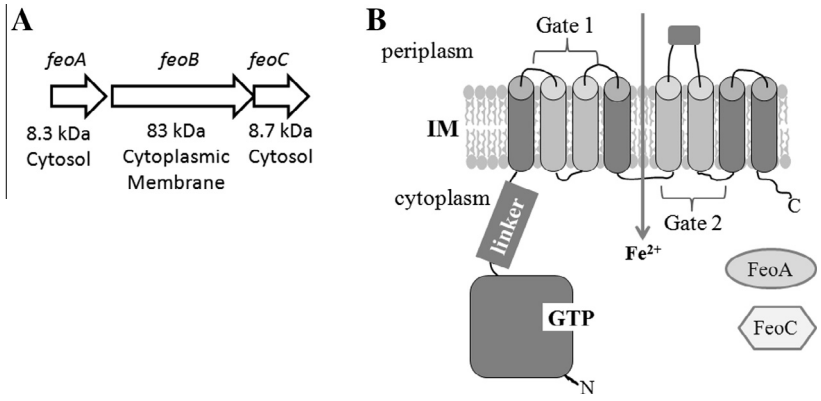

Fig. 1. (A) Organisation of the *feoABC* operon of *P. aeruginosa*. The predicted mass and subcellular location of each of the *feo* translation products are indicated. (B) Schematic representation (not to scale) of the proposed structure of the Feo system. FeoB consists of an N-terminal, cytoplasmic GTPase domain, a GDI linker domain and eight putative transmembrane α-helices. The transmembrane domains may form the pore for iron transport. The roles of FeoA and FeoC are not known, however they both have been shown to interact with FeoB from related species [54,55].

stability of the protein over a pH range from 7.0 to 8.0 and in different detergents was also assessed and functional reconstitution of purified protein in liposomes was achieved.

Materials and methods

Materials

Plasmid pET41-a (+) was from Novagen and *E. coli* C41 (DE3) cells were from Lucigen. Super-competent DH5α cells were from Bioline. Protein molecular weight markers, DNA ladders and other molecular biology consumables were supplied by Fermentas unless indicated otherwise. n-Dodecyl-β-D-maltopyranoside (DDM)[1] and NV10 were from Expedeon, $C_{12}E_8$ from Anapoe and Amphipol A8-35 from Affymetrix. Growth media and kanamycin were from Formedium and isopropyl β-D-1-thiogalactopyranoside (IPTG) from Melford Labs. General chemicals were from Sigma unless indicated otherwise.

Cloning of the feoB gene and generation of the D123N FeoB mutant

The *feoB* gene was amplified from genomic DNA of *P. aeruginosa* strain PAO1 (a gift from Martin Welch, University of Cambridge) with Velocity DNA polymerase (Bioline) using primers 5′-GGAATTC**CATATG**ACCGCATTGACCCTCGGCC-3′ and 5′-CGAGCGGGCAG GGAGGATGTCACCTGGTGCCACGCGGTAGTGGG**AAGCTT**GCGCGC-3′. *Nde*I and *Hind*III restriction sites (shown in bold) were included. The PCR generated sequence was restriction digested and cloned into the pET41-a(+) expression vector (Novagen) to yield pFeoBH, which coded for FeoB with a C-terminal 8-histidine tag. The ligated insert was thereafter transformed into *E. coli* DH5α-silver select competent cells. The resulting recombinant construct was confirmed by restriction digestion at the NdeI/HindIII site and verified by nucleotide sequencing.

A D123N mutant of the wild-type FeoB protein was generated by PCR with pFeoBH as template and using primers 5′-GAACATGCT CAACATCGCCCGTAGCCAGCG-3′ and 5′-CGGGCGATGTTGAGCATGT TCAGCGCGACG-3′. The wild-type plasmid DNA template was removed by restriction digestion of the PCR product with *Dpn*I (New England Biolabs). The D123N *feoB* was restriction digested with NdeI/HindIII and ligated into restriction digested pET41a(+) to yield plasmid pD123N-FeoBH. The cloned PCR product was sequenced to ensure that only the intended changes were introduced.

Preparation of inside-out membrane vesicles

Inside out vesicles were prepared essentially as described before [31] with the following modifications. *E. coli C41* (DE3) cells were transformed with pFeoBH or pD123N-FeoBH, plated on LB agar containing 25 μg/ml kanamycin and incubated at 37 °C. Terrific Broth containing 25 μg/ml kanamycin was inoculated with an overnight culture of *E. coli* C41 (DE3) containing pFeoBH or pD123N-FeoBH. Protein production was induced when the culture reached an OD_{660} of 0.4 by the addition of IPTG (0.5 mM) and the culture was incubated overnight at 18 °C. Cells were harvested by centrifugation (6000×*g*, 10 min, 4 °C). The cell pellet was resuspended in 100 mM K-HEPES (pH 7.0) followed by centrifugation (6000×*g*, 10 min, 4 °C). The pellet was resuspended in 100 mM K-HEPES buffer (pH 7.0) in presence of 10 μg/ml DNase. Cells were lysed by three passages through a basic Z 0.75 kW benchtop cell disrupter (Constant Systems) at 0.138 MPa. The lysate was subjected to low-speed centrifugation at 13,000×*g* for 10 min at 4 °C to remove cell debris. The resulting supernatant was then subjected to high-speed centrifugation at 150,000×*g* for 40 min at 4 °C. The pellet containing the inside out (ISO) membrane vesicles was resuspended in 50 mM K-HEPES (pH 7.0) containing 10% (v/v) glycerol to a protein concentration of approximately 50 mg/ml and stored in liquid nitrogen. The protein concentration of the ISO vesicles was determined by the DC Protein Assay (Bio-Rad Laboratories) with BSA as a standard.

Protein purification

ISO vesicles containing FeoB or D123N FeoB were solubilised by shaking at 4 °C for 1 h in a volume of solubilisation buffer (20 mM K-HEPES pH 7.4, 20% glycerol, 500 mM NaCl, 2% DDM, 10 mM imidazole and 10 mM $MgSO_4$) sufficient to produce a final protein concentration of 10 mg/ml. Unsolubilised protein was removed by high-speed centrifugation (150,000×*g* for 40 m at 4 °C). Ni–NTA (Ni^{2+}–nitrilotriacetate) resin (Sigma) was equilibrated by washing with 20 resin volumes of deionized water, gravity sedimentation on ice and resuspended with 5 resin volumes of wash buffer (10 mM K-HEPES pH 7.0, 10% glycerol, 500 mM NaCl, 0.05% DDM, 10 mM imidazole pH 8.0 and 10 mM $MgSO_4$). Solubilised protein was added to the equilibrated resin

[1] *Abbreviations used:* DDM, Dodecyl-β-D-maltopyranoside; IPTG, isopropyl β-D-1-thiogalactopyranoside; Ni–NTA, Ni^{2+}–nitrilotriacetate; PC, phosphatidylcholine; LB, Luria Bertani; TB, Terrific Broth; OG, n-Octyl-β-D-glucopyranoside.