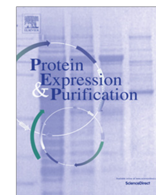
Protein Expression and Purification xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Protein Expression and Purification

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



Expression, purification and spin labelling of the ferrous iron transporter FeoB from *Escherichia coli* BL21 for EPR studies

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ARTICLE INFO

Article history:
Received 19 February 2015
and in revised form 29 May 2015
Available online xxxx

Keywords:
FeoB
Unnatural amino acid
Spin label
EPR
Membrane protein

ABSTRACT

The ferrous iron transporter FeoB is an important factor in the iron metabolism of various bacteria. As a membrane bound GTPase it also represents an interesting evolutionary link between prokaryotic and eukaryotic membrane signalling pathways. To date, structural information for FeoB is limited to the cytosolic GTPase domain and structural features such as the oligomeric state of the transporter in the membrane, and thereby the nature of the transport pore are a matter of constant debate. Recently, EPR distance measurements have become an important tool to investigate such questions in frozen solution. As a prerequisite for these experiments, we designed protocols to express and purify both the cytosolic domain of FeoB (NFeoB) and full-length FeoB from *Escherichia coli* BL21 in purity, quantity and quality needed for EPR studies. Since FeoB from *E. coli* contains 12 native cysteines, we incorporated the unnatural amino acid para-acetylphenylalanine (pAcF) into the protein. We spin labelled the mutant protein using the HO4120 spin label and performed preliminary EPR experiments using *cw*-X-band EPR spectroscopy. Our results provide new insights concerning the oligomeric state of full-length FeoB.
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Introduction

Iron is by mass the most common element on earth and the fourth most common element in the earth's crust. It is also of high biological importance, since many proteins and enzymes depend on iron containing co-factors for catalysis or the transport of electrons or oxygen. Nevertheless, the acquisition of iron poses a problem, because its ferric form (Fe^{3+})[1] is almost insoluble at neutral pH and under aerobic conditions. For this reason, bacteria have evolved sophisticated iron transport systems, including iron chelating compounds (siderophores) and dedicated transporters that import the chelated iron into the cell [1]. Other types of transporters exist for the import of ferrous iron (Fe^{2+}) under anaerobic conditions. An important representative for the latter is the 85 kDa iron transporter FeoB [2]. The protein consists of two major domains: A transmembrane (TM) domain with 7 predicted TM helices and a 30 kDa cytoplasmic domain that is commonly termed "NFeoB" (as in N-terminal domain of FeoB). The latter domain is again split into two subdomains, a GTPase domain and a five-helical bundle. The presence of a GTPase activity in a bacterial membrane protein is interesting in itself, since it might represent an evolutionary link to eukaryotes, where this architecture is used in many signalling pathways [3,4].

A number of crystal structures are available for NFeoB, either with or without the cofactor GTP (or its analogues) [4–9]. Although these structures provide important structural information, the missing transmembrane domains are of course needed to decipher the transport mechanism of FeoB. Based on the crystal packing of the first NFeoB structure from *Escherichia coli* (PDB-ID: 3HYT), a trimeric arrangement of the channel has been proposed, with the transport pore running along the trimer axis [4]. This suggestion is currently under debate because a similar arrangement has not been observed in the crystal structure of NFeoB constructs from *Legionella pneumophila* (PDB-ID: 3IBY), *Thermotoga maritima* (PDB-ID: 3A1S) and *Methanocaldococcus jannaschii* (PDB-ID: 2WJH) [9–11]. Clearly, structural information for the whole channel is needed to clarify this matter.

A lesson from other transporters is that a single crystal structure is not enough to unravel transport mechanisms. This is especially true for proteins such as FeoB where the presence of signalling domains complicates the situation. Thus, alternative methods are needed to complement any crystallographic information. For this reason, we aim to analyse FeoB by EPR methods [12], i.e., to study the oligomeric state of FeoB by PELDOR spectroscopy [13,14]. The same technique will also be helpful to analyse

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[1] *Abbreviations used:* NFeoB, cytosolic domain of FeoB; pAcF, para-acetylphenylalanine; Fe^{3+}, ferric form; Fe^{2+}, ferrous iron; TM, transmembrane; SHQ, Super High Sensitivity; DDM, dodecylmaltoside.

http://dx.doi.org/10.1016/j.pep.2015.05.014
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Please cite this article in press as: G. Hagelueken et al., Expression, purification and spin labelling of the ferrous iron transporter FeoB from *Escherichia coli* BL21 for EPR studies, Protein Expr. Purif. (2015), http://dx.doi.org/10.1016/j.pep.2015.05.014

conformational changes of the protein during transport and upon binding of its cofactor. Here we present protocols for the expression and purification of spin labelled FeoB. Furthermore, the spin labelled proteins are analysed by *cw*-X-band EPR spectroscopy.

Material and methods

Cloning, expression and purification of NFeoB and NFeoB K127K1

The *FeoB* gene from *E. coli* BL21 was cloned into pBADHisTEV (Huanting Liu, University of St Andrews, UK) and a STOP codon was inserted at position 274 of the protein. The plasmid was transformed into *E. coli* C43 (DE3) cells (Lucigen) and typically 3 L of cells were grown at 37 °C in LB medium supplemented with 100 μg/mL ampicillin with shaking (180 rpm). At an OD_{600} of ~0.5, protein expression was induced by adding L-arabinose at a final concentration of 0.2%. The expression was allowed to proceed for 4 h at 37 °C with shaking (180 rpm). For the incorporation of para-acetylphenylalanine (pAcF) into NFeoB, C43 cells containing the pEVOL-pAcF plasmid were transformed with the FeoB–pBADHisTEV construct. Also, 34 μg/mL chloramphenicol were added to the growth medium and 2.5 mM p-AcF were added at the time of induction. Other than that, the expression protocol was identical to that of NFeoB wild-type. Following expression, cells were harvested and the pellet resuspended in buffer A (50 mM Tris–Cl pH 8.0, 50 mM NaCl). A cell disrupter (Constant Systems) was used to disrupt the cells. Cell debris and insoluble material was separated by centrifugation at 20,000 rpm in a Beckman JA 25.50 rotor. The supernatant was then loaded onto a HisTrap FF column (5 mL) using buffer A, washed and eluted with a gradient running from 0% to 100% buffer B (buffer A + 1 M imidazole). The protein was then loaded onto a MonoQ 5/50 column using buffer A and eluted with a gradient running from 0% to 100% buffer C (buffer A + 1 M NaCl). 4 mg TEV protease were added to the sample to cleave the His_6-tag. The sample was then again loaded onto a HisTrap FF column (5 mL). This time, the flowthrough was collected, while uncleaved protein and TEV protease bound to the column material. The flowthrough was concentrated to ~2 mL and loaded onto a Superdex 200 16/60 column using buffer D (20 mM Tris–Cl pH 8.0, 150 mM NaCl). The protein eluted at ~90 mL, corresponding to monomeric NFeoB. Fractions from the gel filtration peak were collected, concentrated to ~60 mg/mL and frozen in 50 μL aliquots. The yield was 13 mg per litre of bacterial culture for NFeoB and 1–3 mg per litre of bacterial culture for NFeoB K127K1.

Cloning, expression and purification of full-length FeoB and full-length FeoB K127K1

For the expression of full-length FeoB, the FeoB gene was cloned into the pBADCHis plasmid (Huanting Liu, University of St Andrews, UK), which was then transformed into C43 cells. Typically, 6 L of cells were grown at 37 °C in LB medium, supplemented with 100 μg/L ampicillin. The cells were grown until the OD_{600} reached ~0.5 and induced with 0.02% L-arabinose. The expression was allowed to proceed for 3–4 h at 37 °C with shaking at 200 rpm. For the incorporation of pAcF into full-length FeoB, C43 cells containing the pEVOL-pAcF plasmid were transformed with the FeoB–pBADCHis construct. Also 34 μg/mL chloramphenicol were added to the growth medium and 2.5 mM pAcF were added at the time of induction. Other than that, the expression protocol was identical to that of FeoB wild-type. Following the expression, cells were harvested and resuspended in buffer A_{fl} (50 mM Tris–HCl pH 8.0, 50 mM NaCl) and lysed using a cell disrupter (Constant Systems) at 30 kPsi. The lysate was centrifuged at low speed (15 min @ 10,000 rcf) to remove cell debris and then 75 min at 75,600 rcf to pellet the membranes. The membranes were extracted using 10 mL buffer B_{fl} (50 mM Tris–HCl pH 8.0, 50 mM NaCl, 1% DDM, 1 mM TCEP) per gram of membranes overnight at RT. On the next day, the extract was centrifuged 75 min at 75,600 rcf and 3 mL Ni^{2+} resin were added to the supernatant. After an incubation time of 1 h at RT with gentle shaking, the Ni^{2+} resin was loaded into a gravity column, washed with 50 mL buffer C_{fl} (50 mM Tris–HCl pH 8.0, 50 mM NaCl, 20 mM imidazole, 0.024% DDM, 1 mM TCEP) and eluted with 4 × 5 mL buffer D_{fl} (50 mM Tris–HCl pH 8.0, 50 mM NaCl, 500 mM imidazole, 0.024% DDM, 1 mM TCEP). The sample was then loaded onto a Superdex 200 10/300 or Superose 6 16/70 column for final purification with running buffer E_{fl} (20 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.024% DDM, 10 mM beta-mercaptoethanol). Yields of ~1.5 mg per litre of bacterial culture were achieved for full-length FeoB. The incorporation of pAcF led to approximately 50% decrease in yield.

Spin labelling

The protein was concentrated to a volume of 2.5 mL and loaded onto a PD-10 column, equilibrated with buffer F_{fl} (100 mM NaH_2PO_4 pH 4.0, 25 mM NaCl, 0.024% DDM, 10 mM beta-mercaptoethanol) or buffer F (100 mM NaH_2PO_4 pH 4.0, 25 mM NaCl) for full-length FeoB and NFeoB, respectively. The protein was then concentrated to ~100 μM and a 10-fold molar excess of HO-4120 (Toronto Research Chemicals) was added. The reaction was left at RT for 12–24 h. Finally, the sample was loaded onto a PD-10 column equilibrated with buffer G (100 mM TES pH 7.5, 150 mM NaCl, 0.024% DDM) in D_2O to remove unreacted label.

EPR spectroscopy

For *cw*-X-band EPR measurements, the spin labelled proteins were concentrated to 10–100 μM. EPR spectra were recorded on an ELEXSYSII E580 X-band EPR spectrometer or an EMXmicro from Bruker, equipped with a Super High Sensitivity (SHQ) resonator for X-band measurements. The samples were measured at room temperature with a microwave power of 2.36 mW (FeoB) or 0.1 mW (NFeoB), a video amplifier gain of 87 dB (FeoB) or 60 dB (NFeoB), a modulation amplitude of 1 G, a time constant of 40.96 ms (FeoB) or 81.92 ms (NFeoB), a conversion time of 41.16 ms (FeoB) or 82.00 ms (NFeoB) and a resolution of 10 points per G. Spectral simulations were performed with EASYSPIN [15].

Results

Selecting a suitable labelling site on the molecular surface of FeoB

Like most proteins, FeoB is diamagnetic and per se "invisible" for EPR. Therefore, in order to study its quaternary structure by PELDOR distance measurements, spin labels have to be introduced into the protein [16]. This is commonly achieved by introducing cysteines at specific sites of the proteins' molecular surface. The cysteines are then spin labelled, for example with the methanethiosulfonate spin label MTSSL [16,17]. FeoB from *E. coli* BL21 is a difficult target for this approach, because it contains 12 native cysteine residues that would have to be removed prior to the insertion of new cysteines. Also, it was not possible to find a suitable cysteine-free homologue of FeoB in sequence databases (http://www-hagelueken.thch.uni-bonn.de/mtsslCysServer/). We therefore decided to follow the approach pioneered by Fleissner et al. [18] and aimed at incorporating the unnatural amino acid pAcF (para-acetylphenylalanine) into FeoB. In order to find optimal spin labelling positions, the FeoB trimer structure (PDB-ID: 3HYN)

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Please cite this article in press as: G. Hagelueken et al., Expression, purification and spin labelling of the ferrous iron transporter FeoB from *Escherichia coli* BL21 for EPR studies, Protein Expr. Purif. (2015), http://dx.doi.org/10.1016/j.pep.2015.05.014

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