Protein Expression and Purification xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Overexpression, purification and biophysical characterisation of *E. coli* MerT

Kien Ly [a], Liam O'Ryan [a,b], Alok K. Mitra [a,*]

[a] University of Auckland, School of Biological Sciences, Private Bag 92019, Auckland, New Zealand
[b] Quantum Detectors Limited, Rutherford Appleton Laboratory, Harwell, Oxford, United Kingdom

ARTICLE INFO

Article history:
Received 1 October 2014
and in revised form 21 November 2014
Available online xxxx

Keywords:
Mercury transport
Membrane protein overexpression
Membrane channel
SEC-MALLS
Circular dichroism

ABSTRACT

Mercury resistance is the most widespread of all anti-microbial resistance occurring in a wide variety of Gram-negative and Gram-positive bacterial genera. The systems that are most studied and best understood are those encoded in mercury resistance (Mer) operons in Gram-negative bacteria. The mercury detoxification functions by the importation of highly toxic Hg^{2+} into cytoplasm and enzymic reduction to volatile Hg^0. MerT is a small (13 kDa) inner membrane protein involved in mercuric ion transport system. We have overexpressed recombinant 6His-tagged MerT from *Escherichia coli* in a native folded form and purified it to homogeneity in n-dodecyl-β-D-maltopyranoside (DDM) by immobilized metal affinity chromatography (IMAC). Circular dichroism showed that the protein is largely α-helical. Size-exclusion chromatography (SEC) in a variety of detergents showed that the protein exists in a multiple of oligomeric states as also confirmed by SEC coupled with multiple-angle light scattering.

Introduction

Mercury is toxic to all living species, as it uses protein thiol groups to form complexes and also can substitute for essential metals e.g. zinc and iron. The methylated toxin i.e. CH3HgX can cross membranes e.g. the blood–brain barrier leading to bioaccumulation of methylmercury and therefore is a considerable environmental concern [1]. Bacteria have evolved a variety of means, generally referred to as resistance mechanisms, for dealing with heavy metals including the many forms of mercury. Plasmid encoded resistance to mercury is as common as the antibiotic resistances and can be found in most bacterial genera [2]. The Gram negative system is one of the most studied examples of plasmid determined mercury resistance (Mer).[1] It contains a narrow spectrum locus encoded by Tn501 and Tn21 and the broadspectrum locus encoded by the IncM plasmid R831b. The Tn501 plasmid contains the structural genes for mercuric reductase MerA and the transport proteins MerT and MerP, flanked by MerR and MerD, which are involved in regulation of expression of the structural genes in response to mercuric salts. Hg^{2+} crossing into the periplasm is taken up by the soluble periplasmic Hg^{2+} scavenging protein (MerP) and then transported into the cytoplasm by one or more inner membrane-spanning proteins (MerC, MerE, MerF, and MerT) [2–7]. Subsequently Hg^{2+} is delivered to MerA, the cytoplasmic mercuric reductase, a multi-domain enzyme that reduces Hg^{2+} to volatile Hg^0. The mechanism of transport of Hg^{2+} across the bacterial membrane mediated by MerC, MerE, MerF or MerT has been well studied [8–11].

The focus of our study, MerT, is located in the inner membrane. MerT, is a small (13 kDa), basic (pI 9.50), very hydrophobic protein. MerT shows limited homology to HisM, HisQ (Histidine transport proteins) and MalF (maltose transport system), which are all dependent upon periplasmic binding proteins for function. Secondary structure predictions implicate 3 α-helical TM (transmembrane) segments in MerT [12]. The Hg^{2+} pathway has been thought to be involved in relay using many thiol pairs. The transfer of Hg^{2+} to MerT from MerP has been postulated to be through coordination scheme contributed by two critical, adjacent cysteines Cys24 and Cys25 in MerT in the first TM segment [12]. After passage through the bilayer, the cation is thought to be "handed over" to a second cysteine pair in the cytoplasmic loop of TM2 and TM3,

* Corresponding author. Tel.: +64 9 923 8162.
E-mail address: a.mitra@auckland.ac.nz (A.K. Mitra).

[1] *Abbreviations used:* Mer, mercury resistance; DDM, n-dodecyl-β-d-maltopyranoside; IMAC, immobilized metal affinity chromatography; SEC, size-exclusion chromatography; MalF, maltose transport system; TM, transmembrane; LDAO, n-dodecyl-n,n-dimethylamine-n-oxide; OG, n-Octyl-β-d-glucoside; C_8E_n, n-Octylpolyoxyethylene; CHAPS, 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate; CYMAL-6, 6-Cyclohexyl-1-hexyl-β-d-maltoside; FC-10, n-decylphosphocholine; FC-12, n-dodecylphosphocholine; CD, circular dichroism; SEC-MALLS, Size exclusion chromatography multi-angle laser light scattering; CMC, critical micelle concentration.

http://dx.doi.org/10.1016/j.pep.2014.11.016
1046-5928/

eventually transferred to another cysteine pair in the N-terminus of a reductase. Apart from putative role of Cys24 and Cys25, no structural information for MerT exists. In order to initiate a comprehensive knowledge of the mercury transport process, it will be important to reveal the chemical and 3-dimensional structural details of the pathway Hg^{2+} follows through MerT. Towards that end in this work we report overexpression, purification of MerT from *Escherichia coli* in a native folded state and biophysical characterisation of the recombinant protein.

Material and methods

Plasmid construction

The *merT* gene (348 base pairs) was amplified from *E. coli* genomic DNA with the following primers: 5′-CATATGTCTGAACCAAAAA CCGG-3′ and 5′-AAGCTTCAGTAGAAAAATGGCATGA-3′ which contain the restriction site *Nde*I and *Hind*III respectively (underlined). The stop codon, TGA, was also included in front of the *Hind*III site. The PCR product was purified and digested with *Nde*I and *Hind*III and inserted into plasmid pET28b digested with the same restriction enzymes. This introduced a 6× histidine tag and a thrombin cleavage site fused to the N-terminal of *merT*. The final construct, designated pET28b-6×His-MerT, was verified by Sanger sequencing (Centre for Genomics and Proteomics, The University of Auckland).

Protein expression and purification

The plasmid pET28b-6×His-MerT was transformed into BL21 (DE3) cells rendered chemically competent based on a previously published protocol [13]. Transformed cells were picked from a single colony and inoculated in a 50 mL flask containing 10 mL of L-broth supplemented with 50 μg/mL kanamycin. The flask was incubated at 37 °C with vigorous shaking for 16 h. The overnight culture was then transferred into a 2 L baffled flask containing 1 L of auto-induction media (10 g/L tryptone, 5 g/L yeast extract, 100 mM Na^+, 50 mM K^+, 50 mM NH_4^+, 25 mM SO_4^+, 100 mM PO_4^+, 1 mM Mg^{2+}, 0.5% glycerol, 0.05% glucose and 0.2% lactose) [14] supplemented with 100 μg/mL kanamycin and incubated at 18 °C with vigorous shaking (>220 rpm) for 24 h. The culture was harvested by centrifugation (4,500×*g*, 30 min, 4 °C). The cell pellet was resuspended in ice cold lysis buffer (10 mM Tris–Cl pH 8.0, 150 mM NaCl) supplemented with Universal protease inhibitor cocktail (Roche) at volume 4 times the wet cell paste. Typically, 1 L of culture yielded ∼5 g of cell.

The resuspended cells (5 g) were disrupted by sonication for 10 min on an ice bucket. A cycle of 0.5 s on/off at ∼60 W was used. The cell lysate was centrifuged (4,500×*g*, 30 min, 4 °C) to pellet debris and unbroken cells. The supernatant was recovered and centrifuged (100,000×*g*, 1 h, 4 °C) to pellet the membrane. The membrane pellet was gently resuspended in 10 mL ice cold buffer (25 mM sodium phosphate pH 7.4, 500 mM NaCl), stored at −80 °C or used immediately for solubilisation. This 10 mL suspension of membrane pellet was supplemented to a volume of 20 mL in final concentration of 25 mM sodium phosphate pH 7.4, 10 mM imidazole, 500 mM NaCl, 10% glycerol, 5 mM TCEP and 2% DDM (n-dodecyl-β-D-maltopyranoside (Anatrace)). The mixture was incubated at 4 °C for 1 h with gentle mixing and then centrifuged again (100,000×*g*, 1 h, 4 °C).

The supernatant which contained solubilised $MerT_{6His}$ was recovered and purified using a 5 mL HiTrap chelating column (GE). Briefly, the column was charged with 5 mL of 100 mM of $CoCl_2$ solution, washed with 25 mL of deionized water and equilibrated with 25 mL of solubilisation buffer containing 0.05% DDM. Detergent extract containing $MerT_{6His}$ was filtered (0.45 μm filter) and applied onto the column. The column was washed with 35 mL of solubilisation buffer with 0.05% DDM, followed by 25 mL 50 mM imidazole pH 7.0, 150 mM NaCl, 1 mM TCEP, 10% glycerol, 0.05% DDM. $MerT_{6His}$ was eluted in 20 mL of 500 mM imidazole, pH 7.0, 150 mM NaCl, 10% glycerol, 1 mM TCEP, 0.05% DDM.

Size exclusion chromatography

SEC was carried out using a variety of detergents. For this purpose, 50 μL of purified protein (∼2 mg/mL) was incubated for 1 h at room temperature with 50 μL of SEC buffer (50 mM Tris–Cl pH 8.0, 150 mM NaCl, 5% glycerol, 0.5 mM TCEP) containing one of the following detergents at a concentration of 5%: LDAO (n-dodecyl-n,n-dimethylamine-n-oxide), OG (n-Octyl-β-D-glucoside), C_8E_n (n-Octylpolyoxyethylene), CHAPS (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate), CYMAL-6 (6-Cyclohexyl-1-hexyl-β-D-maltoside), FC-10 (n-decylphosphocholine) and FC-12 (n-dodecylphosphocholine). The sample was then injected onto Superdex 200 10/300 GL column (GE) on an AKTA prime system (GE) equilibrated with filtered and degassed SEC buffer containing 0.05% DDM at a flow rate of 0.5 mL/minute. The molecular size of detergent-bound $MerT_{6His}$ was evaluated using standards supplied with the gel filtration calibration kit (GE).

Circular dichroism (CD)

Purified protein was diluted to 0.1 mg/mL in CD buffer (5 mM Tris–Cl pH 8.0%, 0.025% DDM) and transferred to a glass cuvette (10 mm path length). CD analysis was performed on the PiStar instrument (Applied Photophysics) at room temperature (25 °C). The data in the 200–240 nm range was uploaded onto the K2D3 server (K2D3.ogic.ca) in order to quantify elements of secondary structure [15].

Size exclusion chromatography multi-angle laser light scattering (SEC-MALLS)

SEC-MALLS analysis of $MerT_{6His}$ was carried out using Superdex 200 10/300 GL column, on a Dionex HPLC with a PSS SLD7000 7-angle MALLS detector and Shodex RI-101 differential refractive index detector. The column was equilibrated in 2 column volumes of SEC buffer with 0.025% DDM. The flow rate for the protein was 0.5 mL/min and values for UV_{280} absorbance, refractive index and light scattering were recorded. A 2 mg/mL BSA standard (M_w = 66,432 Da) was used to calibrate the detector constant as previously described [16]. The detector constant was calculated from Eq. (1)

$$K_{\text{detector}} = \frac{\Delta LS \times \Delta UV_{280}}{M_{W.BSA} \times A_{280.BSA} \times (\Delta RI^2)} \quad (1)$$

The weight-averaged molecular mass of the protein component in the protein–detergent–lipid complexes were then calculated using Eq. (2).

$$M_{w.MerT} = \frac{\Delta LS \times \Delta UV_{280}}{K_{\text{detector}} \times A_{280.MerT} \times (\Delta RI^2)} \quad (2)$$

Result

The *merT* gene amplified from *E. coli* genomic DNA was successfully cloned into pET28b and the sequence is included in the Supplementary data. The $MerT_{6His}$ protein contains 136 amino acids with a calculated molecular weight of ∼15 kDa and pI of 9.7. It could be extracted from the membrane by DDM. The protein

Please cite this article in press as: K. Ly et al., Overexpression, purification and biophysical characterisation of *E. coli* MerT, Protein Expr. Purif. (2014), http://dx.doi.org/10.1016/j.pep.2014.11.016