RESEARCH COMMUNICATION

Expression and purification of functional recombinant meningococcal transferrin-binding protein A

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Pathogenic bacteria of the genus *Neisseria* have a siderophore-independent iron-uptake system reliant on a direct interaction between the bacterial cell and human transferrin (hTf), a serum protein. In the meningococcus, this uptake system is dependent on two surface-exposed, transferrin-binding proteins (Tbps), TbpA and TbpB. TbpA is highly conserved among meningococcal strains, and is thought to be a porin-like integral protein that functions as a gated channel for the passage of iron into the periplasm. TbpB is more variable in size, lipidated and fully surface-exposed. Given its location on the cell surface, its role in pathogenicity and interstrain sequence conservation, TbpA is currently being regarded for inclusion in a meningococcal vaccine effective against all serogroups. This requires gaining knowledge of the ligand–receptor interactions. In the present study we have optimized a procedure for obtaining purified, functionally active recombinant TbpA at a level and stability necessary for the initiation of such studies.

Key words: iron, membrane protein, *Neisseria meningitidis*.

INTRODUCTION

Neisseria meningitidis, a major causal agent of bacterial meningitis and septicaemia in humans, is a Gram-negative diplococcus and the only bacterium capable of generating epidemic outbreaks of the disease [1]. Iron acquisition is key to the pathogenesis of meningococcal disease. Once the meningococcus has become invasive and reached the bloodstream, the organism must compete for iron with host iron proteins that maintain bacteriostatic levels of free iron within the blood. The organism possesses a repertoire of mechanisms to acquire this essential metal, including direct receptor-mediated uptake from the glycoproteins human transferrin (hTf) and lactoferrin [2].

Iron uptake from hTf is mediated by two proteins, transferrin-binding proteins A and B (TbpA and TbpB) [3]. With a molecular mass of approx. 98 kDa, TbpA also displays a high degree of sequence homology among strains [4]. TbpA is proposed to exist as an integral outer-membrane protein, forming a transmembrane pore through which iron can be internalized into the periplasm [3,5]. TbpB displays much more sequence heterogeneity and molecular-mass variation between strains [6,7]. It is thought to be a bilobal lipoprotein that is fully exposed on the bacterial surface, attached to the outer membrane via an N-terminal lipid moiety [8]. Both TbpA and TbpB are capable of independently binding hTf. The mechanism for iron uptake by the meningococcus displays an exquisite specificity for hTf, in that these Tbps are unable to bind transferrins from different species [9–11]. This may go some way towards explaining the host specificity of *N. meningitidis*.

TbpA possesses significant sequence similarity with two *Escherichia coli* siderophore receptors, FhuA and FepA, the structures of which have been determined [12–14]. Both proteins are composed of a 22-stranded, anti-parallel β-barrel connected via a short linker region to an N-terminal globular domain. The β-barrel resembles the architecture of the well-documented porins, whereas the globular domain folds inside the barrel, completely occluding the transmembrane pore.

Although transmembrane prediction programs suggest TbpA adopts a similar structure to those of FepA and FhuA [15], determination of the crystal structure of TbpA is imperative to obtain knowledge of surface-exposed regions that might interact with hTf. This will help to elucidate the mechanism of iron release from hTf, which is currently unknown, as well as providing important information with regard to developing TbpA as a potential meningococcal vaccine.

Previous studies have long highlighted problems associated with the expression and purification of TbpA. It was either lost during the isolation procedure [16], or functionality was severely reduced as a result of the harsh conditions employed for its isolation [17]. Ala'Aldeen et al. [18] and Boulton et al. [19] have reported methods to purify TbpA from the meningococcal TbpA–TbpB complex, but they obtained only very low yields. Attempts at purifying recombinant TbpA (rTbpA) have been largely unsuccessful. Gonzalez et al. [20] expressed rTbpA from *Actinobacillus pleuropneumoniae*, but a majority of the protein was non-functional and remained located in the cytoplasm, even at moderate expression levels. Other studies that describe the expression of functional rTbpA failed either to yield purified protein [21] or to maintain its stability in solution at concentrations sufficient for crystallization [22]. An optimized procedure for the expression and purification of functionally active rTbpA from the outer membranes of *E. coli* BL21 DE3 cells is described in the present study.

Abbreviations used: HRP, horseradish peroxidase; hTf, human transferrin; mTf, murine transferrin; pTf, pig transferrin, rTbpA, recombinant TbpA; Tbp, transferrin-binding protein.

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MATERIALS AND METHODS

Organism source and growth conditions

The *tbpA* gene was obtained from *N. meningitidis* strain K454 (B:15:P1.7,16) [23], and cloned into a pET-20b plasmid with ampicillin resistance (amp^R; Novagen, Madison, WI, U.S.A.). A 5′ oligonucleotide was designed to encode a 10-histidine tag at the N-terminus of the mature sequence, between the *pelB* signal sequence of pET20b and Glu-25, thus omitting the neisserial signal sequence (N-terminus, 5′-gatatccaccatcatcaccatcatcaccatcatcacgaaaatgtgcaagcc; C-terminus, 5′-gaattcttaaaacttcatttccaa). Automated fluorescence chain terminator sequencing (separated on an ABI 310) was used to check that the histidine tag had been inserted correctly, and that no errors had been introduced into the sequence during PCR. The plasmid was transformed into *E. coli* strain BL21 (DE3): F^- *ompT* $hsdS^B$ *gal dcm* [24], which lacks the *ompT* outer-membrane protease. The bacteria were stored as 10% (v/v) glycerol-stock suspensions at −80 °C.

Extraction and solubilization of TbpA

To produce rTbpA, bacteria were grown in 250 ml flasks containing 50 ml of Luria–Bertani medium (Oxoid, Basingstoke, U.K.) containing 100 mg/l ampicillin at 30 °C for 4 h. 'Starter' culture (5 ml) was then used to inoculate 2 litre flasks containing 500 ml of TB medium (Oxoid) and the above antibiotic. These were incubated at 27 °C, and shaken at 200 rev./min. At the late-exponential growth phase (after approx. 20 h), the culture was harvested by centrifugation in a Beckman JLA 10500 rotor (4800 ***g***, 20 min).

Freshly harvested cells (10 g) were re-suspended in 100 ml of cold buffer A [50 mM sodium phosphate (pH 8.0)/200 mM NaCl/30 mM imidazole/10 mM benzamidine]. Surface components were extracted by the addition of 2% (v/v) Elugent (Calbiochem, San Diego, CA, U.S.A.) at 4 °C, followed by gentle shaking for 4 h. Bacterial debris was removed by centrifugation in a Beckman JA17 rotor (50000 ***g*** for 1 h), and the supernatant was filtered through a 0.45 μm filter.

Purification of TbpA

A Ni^{2+}-chelating Sepharose Fast Flow column (Pharmacia, Milton Keynes, U.K.) was equilibrated with buffer B [buffer A containing 0.05% (w/v) n-dodecyl β-D-maltoside (pH 8.0)]. After loading the solubilized protein extract at 0.2 ml/min and at 4 °C, the column was washed with 10 column-volumes of buffer B. Bound protein was eluted with a 100–500 mM imidazole step gradient in buffer A. Fractions containing rTbpA were collected and pooled. The protein sample was then loaded on to an hTf–Sepharose affinity column (hTf prepared in-house from fresh Cohn fraction IV [25], and coupled with Pharmacia CNBr-activated Sepharose, as described in the manufacturer's protocol) equilibrated with buffer C [50 mM Tris/Hcl (pH 8.0)/500 mM NaCl/0.05% (w/v) n-dodecyl β-D-maltoside] at a flow rate of 0.2 ml/min, 4 °C. Following column washing (10 bed-volumes), protein was eluted using buffer C (adjusted to pH 2.0), and 1 ml fractions were collected into tubes containing 100 μl of 1 M Tris (pH 12.4). Fractions containing purified rTbpA were pooled and reduced in volume to 0.5 ml using Centricon 30 and Centriprep 30 concentrators (Amicon, Stonehouse, Gloucestershire, U.K.). The concentrated sample was passed through a HiPrep Sephacryl S-300 (16/60) gel-filtration column (Pharmacia) equilibrated with buffer D [25 mM Tris/HCl (pH 7.5)/200 mM NaCl/1 mM EDTA/0.02% (w/v) NaN_3/0.1% (v/v) *N*,*N*-dimethyldodecylamine-*N*-oxide] at 0.5 ml/min.

Nitrocellulose-based hTf-binding assays and electrophoresis

A nitrocellulose-based Tf-binding assay (dot-blot) was used primarily to ensure correct functionality and species specificity of rTbpA during the above purification protocol. Serial dilutions (1:3) of protein sample were prepared in PBS/0.05% (v/v) Tween 20 (PBS-T_{20}), 3 μl of which were dried on to a nitrocellulose membrane (Bio-Rad, Hemel Hempstead, Herts., U.K.). Remaining active sites were blocked by washing the membrane in PBS-T_{20}/3% (w/v) BSA, followed by 3 × 5 min washes in PBS-T_{20}. Functionality was assessed by probing the membrane with hTf–horseradish peroxidase (HRP) conjugate (hTf–HRP; Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.) (1 μg/ml) in PBS-T_{20} for 1 h with gentle shaking. Species specificity was assessed by probing the membrane with either murine transferrin (mTf)–HRP conjugate (Jackson Immunoresearch Laboratories; 1 μg/ml), or hTf–HRP in the presence of pig transferrin (pTf; 1 μg/ml), both in PBS-T_{20} for 1 h with gentle shaking. Bound hTf–HRP or mTf–HRP was visualized after 3 × 5 min washes with PBS by the addition of PBS containing 4-chloronaphthol (5 μg/ml) and 0.05% (v/v) H_2O_2. Sample purity was determined by SDS/PAGE.

RESULTS AND DISCUSSION

Protein purification

The present paper outlines an optimized procedure for the expression and isolation of stable, functionally active rTbpA in quantities sufficient for biochemical investigation. Nucleotide sequencing indicated that the 10-residue histidine tag had been successfully inserted into the N-terminus of the protein, and that the correct gene sequence was being expressed. Elugent was employed in the initial detergent extraction step because of its efficiency in solubilization of functionally active TbpA and low cost. This was an important feature due to the low protein expression levels seen in this system, and hence the large quantity

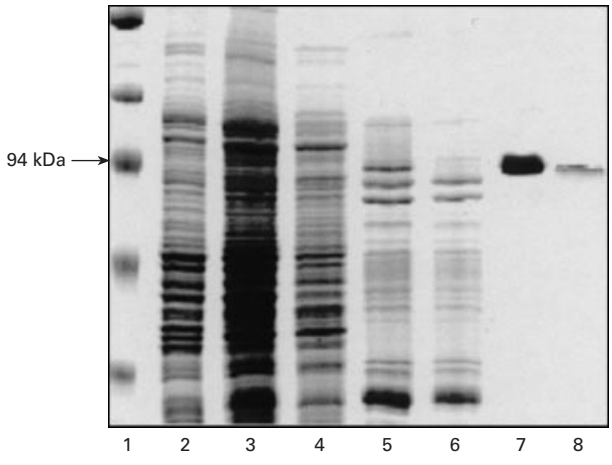

Figure 1 SDS/PAGE gel showing purification of TbpA at various stages in the protocol

Lane 1, broad range markers (Bio-Rad); lane 2, whole-cell lysate (D_{600} 0.6, boiled for 10 min); lane 3, Elugent extract supernatant; lane 4, metal-affinity column flow-through; lane 5, metal-affinity-column pooled fractions; lane 6, hTf–Sepharose column flow-through; lane 7, hTf–Sepharose column pooled fractions (pure rTbpA); lane 8, wild-type TbpA standard from strain K454, kindly provided by Dr A. Gorringe (Centre for Applied Microbiology and Research, Salisbury, U.K.).

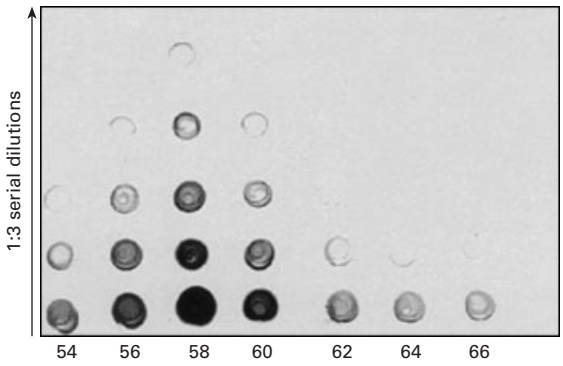

Figure 2 hTf-binding dot-blot analysis, showing location of rTbpA collected from gel filtration column

Lane numbers represent eluted fractions (1 ml).

of cells required to provide sufficient material. Dot-blots indicated that the use of Elugent enabled a large proportion of rTbpA to be extracted from outer membranes.

The initial metal-affinity chromatography step was seen to remove the majority of contaminant proteins within the extraction supernatant (Figure 1, lane 5). No rTbpA was observed in the flow-through, as determined by dot-blot analysis, indicating that the histidine tag was sufficiently exposed to allow efficient and complete binding of the protein to the metal-chelate matrix. A single peak was observed during the elution step, corresponding to an imidazole concentration of approx. 200 mM. Contaminants observed to elute at this imidazole concentration were proposed to possess surface-exposed histidine residues, accounting for their affinity for the metal-chelate column. rTbpA was isolated to purity following affinity chromatography, as revealed by SDS/PAGE analysis (Figure 1, lane 7).

Purified rTbpA was found to be functionally active following gel filtration, as determined by dot-blot assay (Figure 2). Dot-blot studies also revealed that the recombinant protein maintained its species specificity, since binding to hTf–HRP conjugate was not inhibited by the presence of pTf, and it displayed no affinity for a mTf–HRP conjugate (results not shown). Dot-blot analysis of the fractions collected from a minor, earlier peak of the gel-filtration column indicated that these also contained functionally active rTbpA, which migrated to the correct position in SDS/PAGE gels. This earlier peak might represent evidence to suggest that TbpA forms dimers in lipid bilayers, as proposed by Boulton et al. [26], or might be a result of aggregation during the gel-filtration step. Absorbance of the final sample at 280 nm indicated that 1 mg of protein was purified from 1 litre of cell culture. rTbpA concentrations of up to 16 mg/ml have been obtained using Amicon concentrators, with the protein remaining soluble and functionally active following prolonged storage at 4 °C.

Work is currently in progress to grow X-ray-diffraction-quality crystals of rTbpA in order to obtain its three-dimensional structure. The physicochemical properties of the recombinant protein are also being investigated, ahead of the use of site-directed mutagenesis to explore structure–function relationships in TbpA.

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