Protein Expression and Purification 83 (2012) 198–204

Contents lists available at SciVerse ScienceDirect

Protein Expression and Purification

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

Purification, refolding and characterization of the trimeric Omp2a outer membrane porin from *Brucella melitensis*

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ARTICLE INFO

Article history:
Received 4 January 2012
and in revised form 29 March 2012
Available online 17 April 2012

Keywords:
Outer membrane protein
Porin
Brucella melitensis
Refolding
Trimer
Biophysics

ABSTRACT

Brucella melitensis is a gram-negative bacteria known to cause brucellosis and to produce severe infections in humans. Whilst *brucella's* outer membrane proteins have been extensively studied due to their potential role as antigens or virulence factors, their function is still poorly understood at the structural level, as the 3D structure of *Brucella* β-barrel membrane proteins are still unknown. In this context, the *B. melitensis* trimeric Omp2a porin has been overexpressed and refolded in *n*-dodecyl-β-D-maltopyranoside. We here show that this refolding process is insensitive to urea but is temperature- and ionic strength-dependent. Reassembled species were characterized by fluorescence, size-exclusion chromatography and circular dichroism. A refolding mechanism is proposed, suggesting that Omp2a first refolds under a monomeric form and then self-associates into a trimeric state. This first complete *in vitro* refolding of a membrane protein from *B. melitensis* shall eventually lead to functional and 3D structure determination.

Introduction

Brucella melitensis is a gram-negative bacteria responsible for ovine and caprine brucellosis, and among *Brucella* species it produces the most severe infections in humans [1]. Although these bacteria are not particularly host specific, three species (*Brucella abortus, melitensis*, and *suis*) show host preference and diversified pathogenicities for humans [2]. The *Brucella* outer membrane (OM)[1] and associated proteins (OMPs) are studied to find immunogenic and protective antigens for potential diagnostic and vaccinal applications. A class of OMPs (called porins), controls the diffusion through the OM of small hydrophilic molecules [3,4] including drugs, nutrients, and other essential substances supporting growth [5]. The permeability of the OM depends on the pore diameter and the number of porins per cell [6]. *B. melitensis* OMPs extracted with detergents have been identified as group 2 and 3 proteins corresponding to 37 kDa and 26 kDa molecular masses species, respectively, [7,8]. Group 2 proteins were further identified as porins [9]. Among these, Omp2a and Omp2b (from the *Omp2* locus, 85% sequence identity) are 39 kDa general diffusion pores topologically predicted to be organized as trimeric 16-stranded β-barrels [10], but their exact functions remain unknown.

Due to their embedment in the lipid bilayer, membrane proteins are challenging targets in structural biology [11–13]. They first need to be extracted from the membrane and are often unstable. This results in severe difficulties at the expression, solubilization, purification, refolding and crystallization steps. Significant problems upon overexpression of OMPs in living cells arise from overloading and blocking essential components of the cytoplasmic addressing and translocation system [14]. Furthermore, altered membrane properties induced by a massive insertion of recombinant OMPs can cause cytotoxic effects [15]. In *Escherichia coli*, an usual strategy to overcome these drawbacks is the expression in inclusion bodies (IB) [16]. Though the OMPs produced in cytosolic IB adopt non-native conformations, their non-toxicity to the cell and their protection against degradation by proteolysis are obvious benefits [14]. In many cases, expression in IB boosts the OMP production up to several thousand-fold, which is particularly interesting for structural investigations. The bottleneck of this method is to obtain functional OMPs through efficient refolding protocols [17] and the crucial issue is to find the conditions leading to a minimal disturbance of the protein physical environment. Thus, in order to mimic membrane's properties, detergents have to be used from the production stage to their crystallization [18].

To the best of our knowledge, no 3D structure of porins from *Brucella* has been determined so far. As a consequence, solving

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[1] *Abbreviations used:* OM, outer membrane; OMP, outer membrane protein; IB, inclusion body; IPTG, isopropyl β-D-1-thiogalactopyranoside; DDM, *n*-dodecyl-β-D-maltopyranoside; SDS, sodium dodecyl sulfate; SDS/PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CMC, critical micellar concentration; RT, room temperature; IS, insertion sequence.

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http://dx.doi.org/10.1016/j.pep.2012.04.003

the Omp2a X-ray structure would be a key result [19]. Furthermore, once the Omp2a structure is known, a model of the Omp2b porin, another porin encoded by the same locus [20], shall be proposed. In that context, a high-yield Omp2a refolding protocol was set up by optimizing several factors such as urea concentration, temperature, time of refolding, ionic strength or protein concentration. Biophysical characterizations using intrinsic tryptophan fluorescence, size-exclusion chromatography, and circular dichroism (CD), are brought into play to support a two-step mechanism of Omp2a refolding.

Materials and methods

Bacterial strain and growth

Cells of *E. coli* BL21 (DE3) carrying pLysS and pET2a plasmids, containing the *omp2a* coding sequence in which the insertion sequence (IS) the 22 first codons were removed (Fig. 1) as previously reported for Omp2b overproduction [32], were grown in LB medium at 37 °C with constant shaking. Log cultures (OD 0.6) of 200 ml were stimulated with IPTG (0.2 μg/ml) for three hours. Cells were then harvested by centrifugation at 4000 rpm for 30 min, and the resulting bacterial pellets were stored at −20 °C.

Overexpression and non-native purification of Omp2a

The bacterial pellet (corresponding to a 200 ml culture) was thawed and treated with 8 ml of TEN lysis buffer (50 mM Tris–HCl pH 8, 1 mM EDTA, 17 mM NaCl, 125 μM PMSF, 250 μg/ml lysozyme) for 20 min at 25 °C. Harvested cells were further broken by addition of 10 mg of sodium deoxycholate for 60 min at 37 °C with constant shaking, and 2 mg of DNase I (Sigma AMPD1-1KT) for 60 min at 25 °C. The suspension was then centrifuged at 14,000*g* for 20 min at 4 °C. The resulting pellet (containing IB) was twice solubilized using a washing buffer (2 M urea, 20 mM Tris–HCl pH 8, 500 mM NaCl, 2% Triton X-100) and centrifuged at 14,000*g* for 20 min at 4 °C. The inclusion bodies (IB) were solubilized with 8 ml of TEN buffer (50 mM Tris–HCl pH 8, 1 mM EDTA, 17 mM NaCl, 8 M urea). The solubilized proteins were then applied onto an anion-exchange DEAE column (1.5 × 5 cm) previously equilibrated with 25 ml of buffer A (50 mM Tris–HCl pH 8, 17 mM NaCl, 8 M urea). Omp2a was eluted with a 50 ml linear gradient of NaCl from 17 to 500 mM whereas the protein profile was further analyzed using SDS–PAGE. Fractions containing 39 kDa proteins were then pooled and stored at 4 °C. The protein concentration was estimated using the Nanodrop® system by measuring the A_{280} ($\varepsilon_{calculated}$ = 85 300 M^{-1} cm^{-1}, calculated from ProtParam [51]).

Omp2a refolding in n-dodecyl-β-D-maltopyranoside

To refold Omp2a, the protein (1 mg/ml) solution buffer (250 mM NaCl, 50 mM Tris–HCl pH 8 and 8 M urea) was exchanged on a PD-10 column (Amersham Biosciences) to reduce the urea concentration buffer either to 2 M urea in sections Omp2a refolding in *n*-dodecyl-β-D-maltopyranoside and Optimization of Omp2a refolding or to 0 M urea next section Optimization of Omp2a refolding. Protein sample was then diluted 1:1 in a refolding solution (50 mM Tris–HCl pH 8, 250 mM NaCl, 1.2 mM *n*-dodecyl-β-D-maltopyranoside (DDM) (10 times the critical micellar concentration, CMC), and sonicated 3 × 5 min in an ultrasonic bath (Bandelin from Sonorex) at 25 °C to optimize micelle formation (internal results). The protein solution was incubated at room temperature. To stop the refolding reaction, samples were stored at −20 °C.

SDS–PAGE

Samples (20 μl) were loaded on 15% acrylamide SDS–PAGE gels without boiling. After electrophoresis, the gels were stained with Coomassie Blue or silver nitrate and digitally scanned. Densitometry was performed using Image J software. The linear regions in the densitometry profile were determined by measuring the density of standards with known protein amounts. The refolded fraction was estimated by dividing the intensity of the trimer band by the sum of the intensities of both monomeric bands (see main text for a detailed description of bands). The error bars associated with each data point represent the standard deviation of three independent experiments.

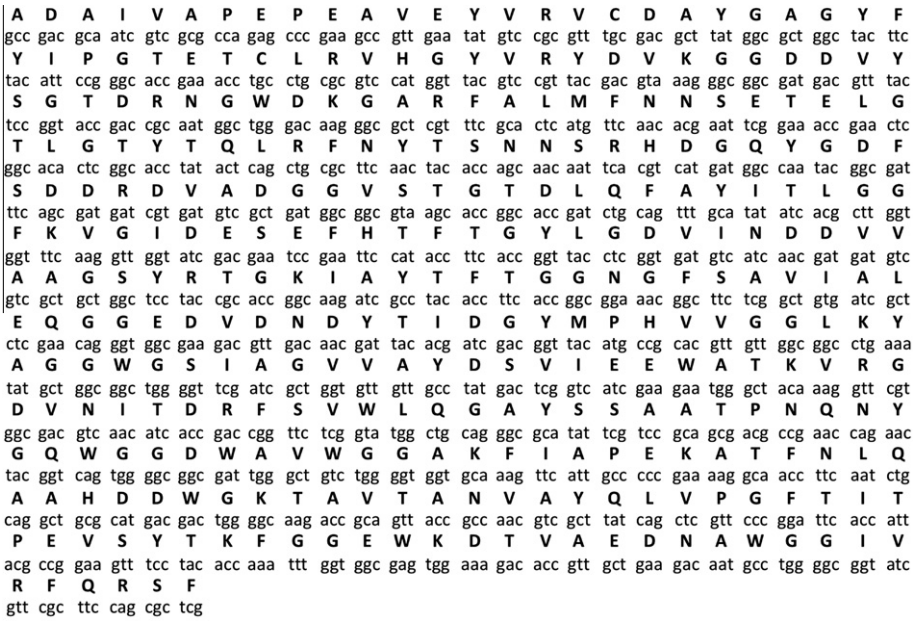

Fig. 1. Nucleotide and amino acids sequences of the Omp2a protein. *Omp2a* locus was inserted in a pET2a plasmid. The 22 first codons were removed, as previously reported for Omp2b overexpression [32].