American Journal of

Biochemistry and Molecular Biology

ISSN 2150-4210

Academic
Journals Inc.

www.academicjournals.com

American Journal of Biochemistry and Molecular Biology 1 (2): 185-199, 2011
ISSN 2150-4210 / DOI: 10.3923/ajbmb.2011.185.199

Quaternary Structure of Omp85/YaeT/BamA of *Yersinia pestis*

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ABSTRACT

The aim of the study was to investigate quaternary structure of Omp85 outer membrane protein of *Y. pestis*, because of its potential use as a potential therapeutic target. Bacterial outer membrane proteins are suitable targets for the antimicrobial drugs and vaccine development. In Gram-negative bacteria, outer membrane protein, Omp85 is an integral part of the protein machinery. Inhibiting and disabling of Omp85 has a negative effect on the secretion of virulence factors, as well as a bactericidal effect. Previous research has identified components of the Omp85 complex and has started to yield structural insights into Omp85 family proteins, the quaternary structure of Omp85 itself remains under investigation. We present a quaternary structure study of *Yersinia pestis* Omp85 *in vitro* and *in vivo* using multiple approaches. Full-length Omp85, purified without denaturation, is shown to form monomers, dimers and tetramers *in vitro* using cross-linking and sedimentation studies. *In vivo* studies, using Western blot of outer membranes resolved on semi-native LDS-PAGE, indicate that Omp85 formed tetramers. Our studies contribute to a better understanding of Omp85 structure and mechanism. The applied relevance of this study has potential use in the development of new vaccines and antimicrobials, which may be potent weapons in the fight against bacterial infections.

Key words: Omp85/YaeT/BamA, outer membrane protein, plague, quaternary structure, Gram-negative pathogen, *in vitro* structure determination, *in vivo* structure determination

INTRODUCTION

Plague is one of the oldest identifiable human diseases and is certainly one of the most feared (Alvarez and Cardineau, 2010; Williamson, 2009). Currently, it is one of only three internationally quarantined diseases. The causative agent of plague is a highly pathogenic Gram-negative bacterium, *Yersinia pestis*. It already caused three human plague pandemic with millions of deaths each (Williamson, 2009; Keim and Wagner, 2009). Depending on the mode of transmission, human plague can exist in three forms: bubonic, pneumonic and septisemic (Alvarez and Cardineau, 2010; Williamson, 2009).

Thousands of human plague cases worldwide are reported annually (Alvarez and Cardineau, 2010). Isolation of *Y. pestis* strain resistant to all antibiotics currently prescribed against this infection highlights the danger of this bacterium (Keim and Wagner, 2009). Geographical distribution of human plague cases is expanding rapidly (Alvarez and Cardineau, 2010). Due to increase in the number of human plague cases since early 1990s, the World Health Organization (WHO) re-classified *Y. pestis* as a re-emerging infectious disease (Alvarez and Cardineau, 2010; Williamson, 2009).

Because *Y. pestis* can be spread through aerosol and lead to an outbreak, the United States Government has classified this bacterium as a possible bioterrorism weapon,

Fig. 1: Sequence alignment of Oma87 of *Pasteurella multocida*, D15 of *Haemophilus influenzae* and Omp85 of *Y. pestis*

current vaccine against *Y. pestis* is not available to public, requires booster shots, has a high risk for adverse reactions and does not protect against the pneumonic form of plague (Alvarez and Cardineau, 2010; Williamson, 2009).

Due to rise in antimicrobial resistance, increase in human plague cases and the lack of adequate vaccine, there is a critical demand to develop new and effective vaccines and antimicrobial drugs against *Y. pestis*.

The use of bacterial outer membrane proteins represents one of the prospective targets in the development of protective vaccine and antimicrobials against bacterial diseases. As a consequence, it is important to understand the structure and function of these proteins.

Since many bacterial proteins important for virulence are secreted via the secretion pathways containing the outer membrane components, it would be desirable to design antimicrobial drugs which are capable of inhibiting these processes. Omp85 appears to be a suitable target because it plays an important role in outer membrane protein biogenesis. Moreover, it is itself an outer membrane protein and is exposed to the extracellular space making it a potential target for vaccine and antimicrobial drug development (Bos and Tommassen, 2004; Doerrler and Raetz, 2005; Su *et al*., 2010).

Omp85 vaccines are promising because Omp85 of *Y. pestis* shares high sequence homology to protective antigens Oma87 (45%) of *Pasteurella multocida* and D15 (43%) of *Haemophilus influenzae* (Huang *et al*., 1992). The Omp85 C-terminal domain resembles those of Oma87 and D15 even more. There is 46.1% sequence identity between the C-termini of Omp85 and Oma87 and 48.5% between the C-termini of Omp85 and D15 (Fig. 1). The recent finding that Omp85 of *Burkholderia pseudomallei* exhibits a protective efficacy in mice further highlights the promise Omp85 holds as a vaccine target (Su *et al*., 2010).

Bacterial proteins synthesized in the cytoplasm can cross the inner membrane by means of a Sec translocase pathway, a Twin Arginine Transport (TAT) system, or a Type I secretion. The

detailed process of translocation across the outer membrane and protein folding in the absence of energy source such as ATP remains unclear. Omp85 family of proteins has been revealed and their role in the outer membrane proteins oligomerization and their insertion has been explored. New report found evidence of association between Omp85 complex and the energy generation system Nar/Fdh-N (Pan *et al.*, 2010). More studies will need to be carried out to conform this finding. It has been found that Omp85 is essential for cell viability and the integration of outer membrane proteins into the bacterial membrane, some of which play an important role in virulence (Ieva and Bernstein, 2009; Voulhoux *et al.*, 2003; Doerrler and Raetz, 2005; Bodelon *et al.*, 2009). Omp85 family proteins are conserved among the bacteria, the mitochondria and the chloroplasts. In bacteria, Omp85 is an integral part of hetero-oligomeric protein complex which is essential for the incorporation of OMPs into the outer membrane (Jacob-Dubuisson *et al.*, 2009) and is the only protein in the complex to completely traverse the outer membrane. Recently, the first details of the Omp85-mediated translocation mechanism started to come into focus. The precursor polypeptide with the N-terminal signal sequence is recognized and transported across the inner membrane by the Sec machinery in an ATP-dependent manner. After the translocation is complete, the signal peptidase cleaves the signal sequence releasing the mature polypeptide into the periplasm where it is stabilized by periplasmic chaperones (Bos and Tommassen, 2004). Then a substrate protein interacts with the multi-protein machinery containing Omp85. This leads to the substrate protein folding, oligomerization and its release into the bacterial outer membrane. To better understand the essential process of protein insertion into the outer membrane, it is important to determine structural details of key proteins involved.

The structure of FhaC, one member of Omp85 super-family of proteins, has been elucidated (Clantin *et al.*, 2007). Numerous models have been proposed. They all agree that Omp85 forms a two-domain structure comprised of an N-terminal periplasmic domain and an integral outer membrane C-terminal domain. The N-terminal domain, whose structure has been solved (Kim *et al.*, 2007; Gatzeva-Topalova *et al.*, 2008; Knowles *et al.*, 2008), contains five chaperone-like POTRA (Polypeptide-transport-associated) repeats which are thought to serve as chaperones for the incoming substrate OMPs (Bos and Tommassen, 2004). This domain is required for Omp85 oligomerization beyond dimers *in vitro*, but not for the ion-conducting channel function (Bredemeier *et al.*, 2007). The models predict that the C-terminal forms a 12-stranded or 16-stranded transmembrane β-barrel in the outer membrane (Voulhoux *et al.*, 2003; Jacob-Dubuisson *et al.*, 2009). Many details of Omp85-mediated folding and insertion process still remain obscure despite its vital role in bacterial viability and virulence Numerous studies have been conducted with structure, function and structure-function relationship of POTRA domains (Delattre *et al.*, 2010; Koenig *et al.*, 2010). However, structural details of C-terminal β-barrel and the protein as a whole are still under debate.

Earlier studies have shown that Omp85 forms oligomers, whereas others found that it forms monomers or dimers (Robert *et al.*, 2006; Kim *et al.*, 2007; Knowles *et al.*, 2008; Clantin *et al.*, 2007). The crystal structure study undertaken by Kim and colleagues involved only a part of periplasmic domain containing POTRA domains and concluded that Omp85 exists as a dimer. It did not account for the impact of the P5 POTRA or the transmembrane domain on the oligomerization equilibrium of the protein. Authors used Blue-Native PAGE technique to resolve Omp85 complex (Kim *et al.*, 2007). Their results, however, do not exclude a possibility that the complex might dissociate in process, what they detected was oligomeric Omp85 instead of monomeric multi-protein complex. Even though the authors found protein dimers in their crystal structure but thought that they are physiologically irrelevant.

Subsequent crystal structure studies of a more complete periplasmic domain by Gatzeva-Topalova and colleagues and NMR studies by Knowles and colleagues argued that Omp85 exists as a monomer (Knowles *et al*., 2008; Gatzeva-Topalova *et al*., 2008). Both the studies once again only looked at the periplasmic N-terminal and disregarded the effect of the transmembrane C-terminal. Another study with a distant homologue, FhaC, showed that the transmembrane domain exists as a monomer (Clantin *et al*., 2007). However, there are many functional differences between FhaC and Omp85. Moreover, the two proteins are only distantly related (Jacob-Dubuisson *et al*., 2009).

More recent study by Hagan and colleagues found the relative ratio of each kind of protein in the multi-protein Omp85 machinery. The authors highlighted that their study did not exclude the possibility that several of such complexes can come together to enable the formation of Omp85 dimers or tetramers (Hagan *et al*., 2010).

Early studies undertaken by Robert and colleagues suggested that Omp85 forms oligomers, possibly tetramers, *in vitro*. However, the conclusion was based on only a couple of experiments and used Omp85 protein purified from inclusion bodies and re-folded *in vitro* (Robert *et al*., 2006). Taken all these studies together, there is still a debate in the community over the oligomeric state of the Omp85 (Knowles *et al*., 2009).

Present objective is to study natively-purified full-length Omp85 of *Y. pestis* to minimize possible artifacts associated with the process of denaturation and refolding and working with truncated proteins. Our aim is use multiple techniques to determine quaternary structure of Omp85 *in vitro* and *in vivo*. Present study contributes to a better understanding of the Omp85 mechanism and can help to develop new vaccines and drugs against *Y. pestis*.

MATERIALS AND METHODS

Project was undertaken from August 2006 to May 2007 at the University of Houston, Houston, Texas, USA

Cloning of *Y. pestis* omp85: *Y. pestis* omp85 gene was amplified from chromosomal DNA, in 50 μL using 1 μL of 1:10 *Pfu* (Stratagene, La Jolla, CA) to *Taq* polymerase (NEB, Ipswich, MA) mix, 10 mM of each dNTP (NEB, Ipswich, MA), 100 ng of the template chromosomal DNA from KIM strain (courtesy of Yihfen Yen, University of Houston), 10 pmol of the following primers and 5 μL of 10X *Pfu* buffer mix. The sequence of forward primer was TAG GAT CCG ATG AAA AAG and the sequence of reverse primer was GGT ACT CGA GCC AAG TTT TAC CAA TG (MWG Biotech, High Point, NC). PCR was performed using the following program: the initial denaturation at 96°C for 5 min, 30 cycles of denaturation at 96°C for 30 sec, annealing at 56°C for 30 sec, extension at 72°C for 1 min, the final extension at 72°C for 10 min. The PCR product was purified and treated with *BamHI* and *Xho*I (NEB, Ipswich, MA) for 4 h at 37°C and the digested PCR product was gel-purified. The plasmid pET21B (Novagen, Madison, WI) was also digested with *BamHI* and *Xho*I and gel-purified. The *omp85* gene was then ligated into the linearized pET21B plasmid in 2:1 ratio, in 20 μL reaction, in the presence of 10 mM ATP, 1 μL ligase (Promega, Madison, WI) and 2 μL of 10X ligation buffer at 4°C for 16 h. The produced pET21B-Omp85 plasmids were transformed into *E. coli* expression strain BL21. The clone that showed the highest Omp85 expression was sequenced.

Growth conditions for *E. coli* BL21/pET21B-Omp85: Strains were grown overnight at 37°C in LB medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl) in the presence of 0.1 mg mL^{-1} ampicillin (Sigma-Aldrich, St Louis, MO). One milliliter of overnight culture was used to inoculate 1 L of fresh LB medium containing 0.1 mg mL^{-1} ampicillin. The culture was grown at 37°C until the OD_{600} of 0.6. Expression of the Omp85 protein was induced by the addition of 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. Cells were harvested, bacterial outer membranes were isolated and proteins were extracted as described previously (Hritonenko *et al.*, 2006).

Purification of Omp85: After the centrifugation to remove the insoluble material, 2 mM $MgCl_2$ was added to neutralize EDTA. Next, 5 mL of the protein extract containing 0.2 mg of protein was loaded onto 5 mL HisTrap HP affinity column (GE healthcare, Chicago, IL) equilibrated with 20 mM Tris-Cl pH 7.0, 100 mM KCl, 10 mM SB 3-12 (Sigma-Aldrich, St. Louis, MO). Proteins were eluted with linear gradient of imidazole (Sigma-Aldrich, St. Louis, MO) from 0 to 500 mM. The protein elution was monitored at wavelength of 280 nm. To determine purity of the eluted Omp85, 200 μL of each fraction was precipitated with 800 μL of acetone overnight at -20°C. The precipitated proteins were pelleted at 10,000 rpm at 4°C in refrigerated 5417 R Eppendorf centrifuge, F-45-30-11 rotor (Hamburg, Germany). Protein pellets were air dried, resuspended and resolved on 10% SDS-PAGE. Protein bands were visualized with silver staining. Fractions containing pure Omp85 were pooled together and concentrated using 30 K cut off Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA). The protein concentration was determined with a BCA assay (Pierce, Rockford, IL).

Heat modifiability of Omp85: The heat modifiability assay was performed as described by Thanassi and colleagues with some modifications (Thanassi *et al.*, 1998). In short, the purified Omp85 and the purified outer membranes were mixed with the loading buffer containing 0.1, 1, or 2% LDS instead of SDS. The samples were either heated to 100°C for 5 min or kept on ice. All samples were then cooled on ice for additional 5 min and resolved on 5% LDS-PAGE at 4°C at 1 mAmp. The protein bands were visualized with silver salts.

Protease accessibility of Omp85: The native or denatured XL1-Blue/pET21B-Omp85 outer membranes and the purified Omp85 were incubated with trypsin (Sigma-Aldrich, St. Louis, MO). To denature proteins, the samples were mixed with SDS-loading buffer (SDS, β-mercaptoethanol) and boiled for 10 min, followed by immediate cooling on ice for 5 min. Digestion conditions were: 2.8 μg of the purified protein or 0.1 mg of outer membranes were treated with 1 μg of trypsin (Sigma-Aldrich, St. Louis, MO) for 5 min at 4°C in a final volume of 1 mL of 20 mM Tris-HCl buffer (pH 7.5). To terminate digestion, the reaction mixtures were boiled for 5 min and combined with 10 mM PMSF (Sigma-Aldrich, St. Louis, MO). The reactions containing the purified proteins were precipitated with acetone (Sigma-Aldrich, St. Louis, MO) overnight. Nine microliters of the outer membrane digestions and the resuspended acetone precipitates were resolved on a 10% SDS-PAGE. The protein bands were visualized using silver staining.

Cross-linking of the purified proteins OmpU, Omp85 and Tsh_{β}: Purified proteins were dialysed twice against 20 mM HEPES buffer (pH 7.5), 150 mM NaCl, 10 mM SB 3-12 and concentrated using 10 kDa cutoff or 30 kDa cut off Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA). The resulting protein concentration was measured using BCA assay.

DSS, Disuccinimidyl suberate, (Sigma-Aldrich, St. Louis, MO) was dissolved in DMSO (Fisher Scientific, Pittsburgh, PA). DSS and the purified proteins were combined in the DSS:protein ratio of 1:0.92. The resulting mixtures were incubated at RT for 1 h in a final volume of 1 mL of 20 mM HEPES buffer (pH 7.5) (Touze *et al.*, 2004). The reactions were terminated with 20 mM Tris-Cl buffer (pH 7.5) (Touze *et al.*, 2004). Membrane proteins were precipitated with acetone overnight. Proteins were resolved on 10 or 6% SDS-PAGE and visualized with silver staining.

Sedimentation studies of Omp85: Procedure was performed as described before with the following modifications (Xu *et al.*, 2003). Linear 10-30% glycerol gradients were prepared with 20 mM Tris-Cl (pH 7.5), 100 mM NaCl and 2 mM $MgCl_2$. The detergent SB 3-12 was added to obtain 10 mM final detergent concentration in the glycerol gradient for Omp85. Protein samples, in the same buffer as the gradients, but with only 5% glycerol, were layered on the top of the gradients. The following sedimentation markers were used from Sigma-Aldrich (St. Louis, MO): bovine hemoglobin (4.3 S), calf intestinal mucosa alkaline phosphatase (6.2 S), bovine liver catalase (11.15 S). Rho, transcription termination factor (Pinkham and Platt, 1983; Oda and Takanami, 1972; Bear *et al.*, 1988) was used as an additional control. Samples were centrifuged at 40,000 rpm, 20.25 h at 4°C in a Beckman SW 50.1 rotor and each gradient was fractionated into 200-μL fractions. Rho and catalase were detected by light absorbance at the wavelength of 280 nm. Hemoglobin and alkaline phosphatase were detected by standard procedures (Gan and Richardson, 1999; Xu *et al.*, 2003). Omp85 fractions were precipitated with acetone overnight at -20°C, pelleted by centrifugation and resolved on a 10% SDS-PAGE. The protein bands were visualized with silver salts. Standard curve was constructed in Excel 2003.

Anti-His_6 Western blot of BL21/pET21B-Omp85 outer membranes: Isolated outer membranes were treated with 0.1% w/v LDS for 10 min on ice, mixed with loading buffer and resolved on 4% LDS-PAGE at 4°C at 1 mAmp. Once the electrophoresis was complete, proteins in the gel were denatured by wrapping the gel in foil, sealing it in plastic bag and keeping it in water steam for 10 min (Brok *et al.*, 1995). The proteins were electroblotted onto a nitrocellulose filter. The filter was washed in PBS for 5 min, blocked with 0.05 % non-fat milk in PBS for 1 h, washed 3 times in 0.05% Tween 20-PBS and incubated with the anti-His_6 mouse monoclonal antibody conjugated to an alkaline phosphatase (Sigma-Aldrich, St. Louis, MO) 1:2,000 in PBS overnight. The filter was washed 3 times in 0.05% Tween 20-PBS and alkaline phosphatase was developed with color-producing reagents BCIP and NBT (Sigma-Aldrich, St. Louis, MO).

RESULTS

Our objective was a detailed quaternary structure analysis of Omp85 of *Y. pestis*, a causative agent of plague. *Y. pestis* Omp85 gene was cloned and expressed in *E. coli*. The Omp85 protein was extracted and purified from the outer membranes. Then, its native folding was verified using trypsin digestion and heat modifiability. Cross-linking studies showed that Omp85 forms monomers, dimers and tetramers *in vitro*. Omp85 sedimentation study and anti-His_6 Western blot of Omp85 in the outer membranes resolved on semi-native gel demonstrated that Omp85 existed mostly as a tetramer *in vitro* and *in vivo*.

Expression, cellular localization, extraction and purification of Omp85: *Y. pestis* Omp85 gene was successfully amplified, cloned into pET21B-Omp85 and expressed in *E. coli*. The next objective was to extract and purify Omp85 from the outer membranes of *E. coli* BL21/pET21B-

Omp85, without unfolding the protein. Omp85 was extracted from the outer bacterial membranes into detergent micelles using 50 mM Tris-Cl and 1% Triton X-100 detergent. Triton X-100 contributes to the disruption of the lipid bilayer releasing the integral membrane proteins. The detergent micelles are formed around these proteins stabilizing them and preventing their unfolding. The detergent micelles were subsequently separated from the insoluble lipids by centrifugation. Omp85 was purified from the other proteins in the extract using the Ni-affinity chromatography. The protein purity was monitored by SDS-PAGE andOmp85 was found to be pure (Fig. 2).

Verification of native folding of Omp85 after the purification: We carefully designed our purification protocol to avoid the denaturation of Omp85 protein. To ensure that our protein was folded even after the process of purification, we relied on two hallmark properties of the outer membrane proteins, namely: resistance to proteases (Fig. 3) and heat modifiability (Fig. 4). First, Omp85 was tested as a suitable substrate for trypsin protease. For that, the purified outer membranes containing Omp85 were denatured by heat and subjected to trypsin proteolysis. The reaction mixture was resolved on denaturing SDS-PAGE and there were no protein bands corresponding to full-length Omp85 (Fig. 3a, lane 3). This means that Omp85 was a suitable substrate for trypsin. To test whether the native Omp85 was resistant to trypsin treatment, the unheated outer membranes containing Omp85 were subjected to the trypsin treatment.The reaction mixture was again resolved on the denaturing SDS-PAGE. The band corresponding to Omp85 was detected, as marked by the star (Fig. 3a, last lane). However, the intensity of the band (Fig. 3a, last lane) was lower as compared to the Omp85 band in the untreated sample (Fig. 3a, second lane). The comparison of second and last lanes (Fig. 3a) showed that either trypsin was capable of

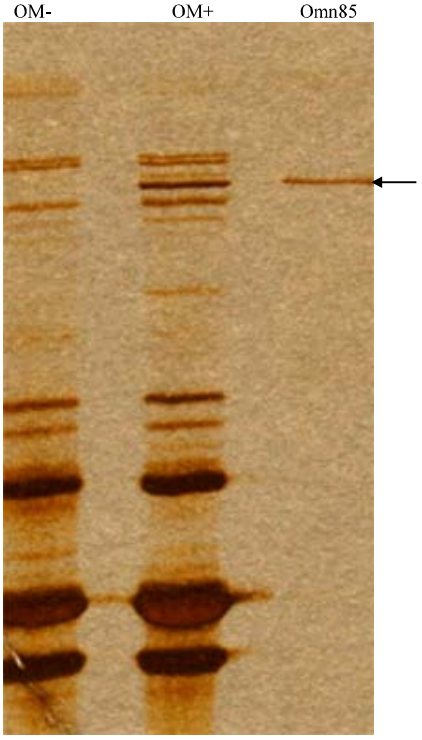

Fig. 2: Omp85 purification from a protein extract using the Ni-affinity chromatography

partially degrading even folded proteins or that proteins in the outer membrane preparation were partially unfolded. This experiment demonstrated that Omp85 was susceptible to trypsin degradation even under native conditions, while Omp85 was still in the outer membrane. This was likely a consequence of Omp85 being a two-domain protein, with only one domain (C-terminal) being protected by the outer membrane.

The same trypsin accessibility experiment was performed after the process of purification. The untreated Omp85 was resolved on a denaturing SDS-PAGE, as a positive control and the band corresponding to a full-length Omp85 was detected at the predicted 89 kDa (Fig. 3b, first lane). When the denatured Omp85 was treated with trypsin and resolved on a SDS-PAGE, as expected, there was no band, because the denatured Omp85 was completely degraded by trypsin (Fig. 3b last lane). When the non-denatured Omp85 was treated with trypsin and resolved on the gel, faint bands of lower molecular mass than the full length of Omp85 were observed. They likely represented the C-terminal domain of Omp85 that forms a ß-barrel which is more resistant to trypsin treatment than the denatured Omp85. The comparison of last lanes of Fig 3a and 3b, showed that Omp85 in the outer membrane preparations is less susceptible to trypsin degradation than the purified Omp85 because of the protection offered by the membrane lipids and other proteins present.

To elaborate and strengthen the trypsin accessibility data of Omp85, a heat-modifiability experiment was performed. It relied on the fact that the outer membrane proteins form ß-barrels which, when folded, result in bands with a lower molecular weight than when they are unfolded by boiling and detergent treatment. In this kind of gel electrophoresis, sodium ion in SDS detergent

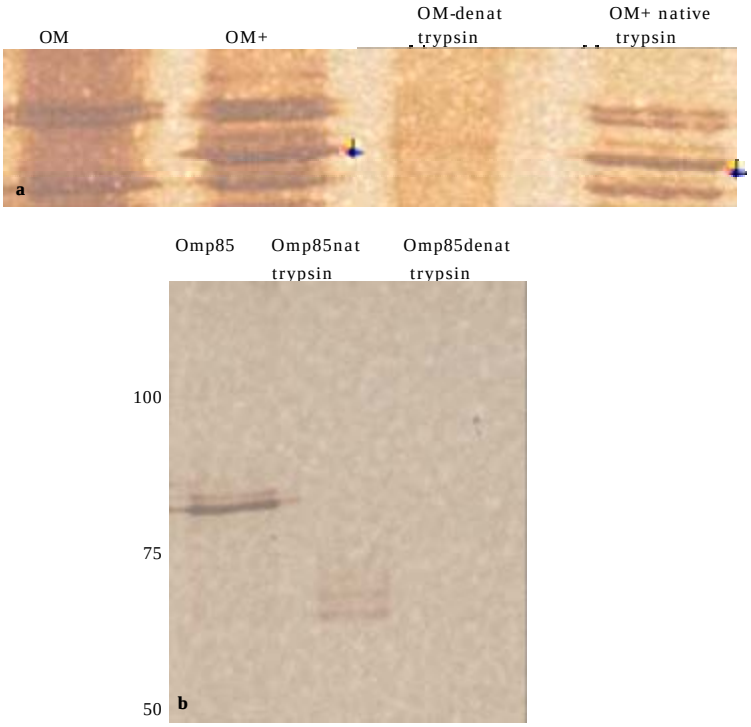

Fig. 3: Trypsin digestion of Omp85 *in vivo* and *in vitro*

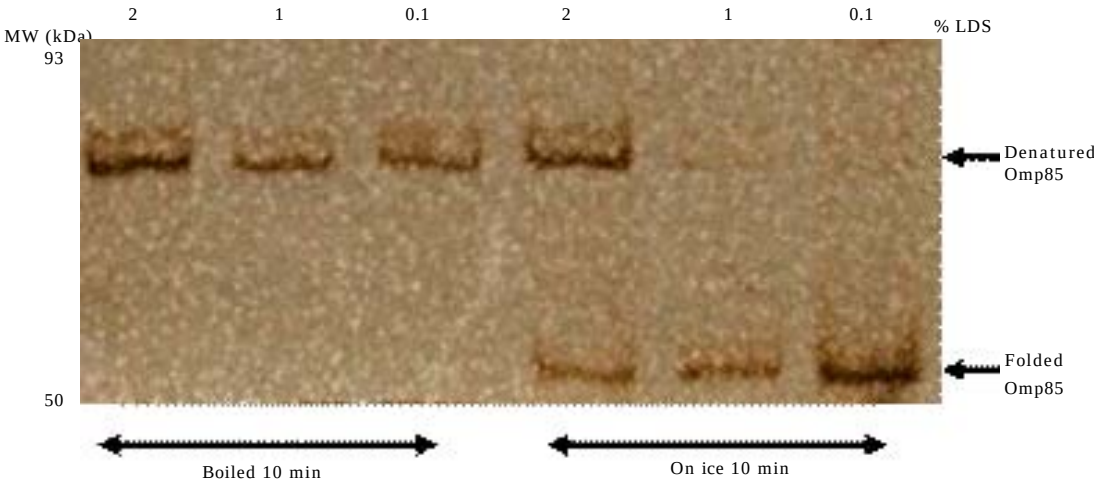

Fig. 4: Omp85 retains its native folding after the purification, based on heat modifiaility experiments

is substituted by lithium ion, because at 4°C SDS precipitates out of the solution and LDS stays soluble (Kubo and Takagi, 1986). Both LDS and SDS interact with proteins in a similar manner. The resulting LDS detergent has a similar effect on proteins as SDS, but does not precipitate at 4°C, the temperature at which the semi-native gel electrophoresis was performed (Moustafa *et al.*, 2003). Moreover, at 4°C, LDS enhances the protein resolution on a gel electrophoresis (Kubo and Takagi, 1986). The sample was not boiled prior to the experiment as in a regular denaturing gel electrophoresis. Instead, Omp85 was incubated on ice with the LDS detergent. Heat modifiability (Fig. 4) experiment showed that the difference in the apparent (50 kDa) and the calculated weight (89 kDa) of native monomer Omp85 is about 40 kDa. In other words, the native Omp85 runs at 56% (50/89) of its calculated molecular weight. Heat modifiability studies of the purified Omp85 show that the 89 kDa Omp85 is natively-folded (Fig. 4). Also, comparison of the three lanes of "on-ice incubation" in Fig. 4, shows that the increasing LDS concentration alone could cause a partial denaturation. This is likely due to the fact that Omp85 is a two-domain protein and only the C-terminal membrane domain is folded into ß-barrel. Another possible explanation is that Omp85 forms a dynamic structure which is more susceptible to denaturation.

Investigation of oligomerization of Omp85 *in vitro*: Once the folding of the purified Omp85 was verified, its *in vitro* quaternary structure was explored using a cross-linking technique. The non-cleavable DSS was chosen as a cross-linker. The purified Omp85 was incubated with DSS. The reaction was terminated by addition of 1 M Tris-Cl and the proteins were precipitated by addition of acetone and overnight incubation at -20°C. As a result, it was found that Omp85 forms monomers, dimers and tetramers (Fig. 5a). Purified OmpU of *Vibrio cholerae*, a kind gift from Dr Delcour, a known trimeric bacterial outer membrane protein, was used as a positive control. It was shown to run as a monomer, dimer and trimer under the same conditions (Fig. 5c). Tsh_{β}, a monomeric bacterial outer membrane protein (Hritonenko *et al.*, 2006) was used as a negative control and was shown to run as a monomer and no additional bands were observed (Fig. 5b).

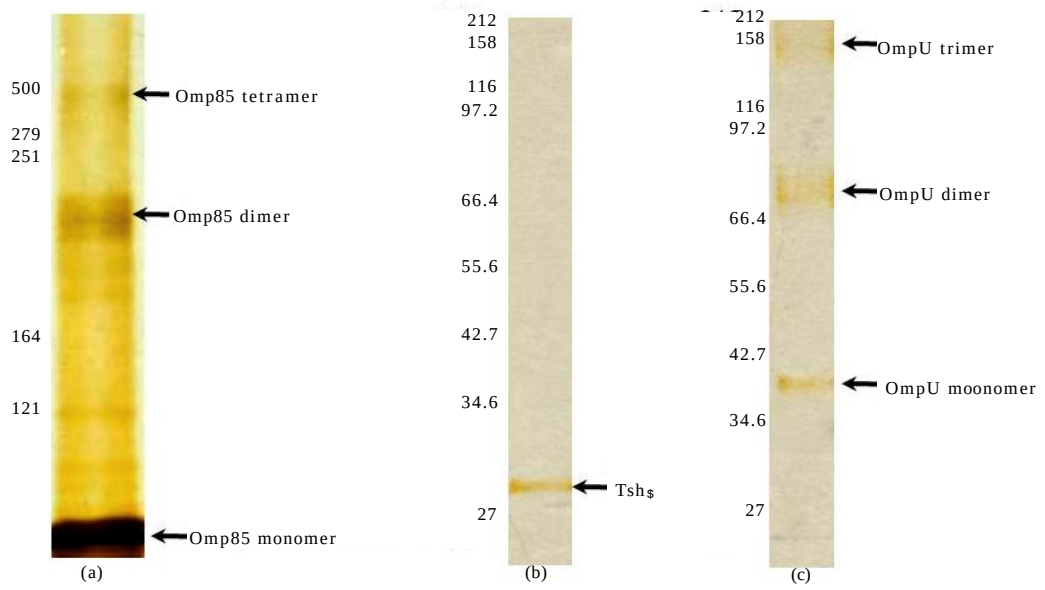

Fig. 5: Cross-linking studies show that the purified Omp85 forms monomers, dimers and tetramers

Ultracentrifugation is a useful tool to detect relatively weak protein associations. We employed this technique to assess the quaternary structure of the purified Omp85. Glycerol gradients are especially helpful for membrane proteins because glycerol has been known to enhance the stability of membrane proteins (Lebowitz *et al.*, 2002). Ultracentrifugation in glycerol gradient was employed to determine the sedimentation coefficient of the purified Omp85. As standards, the following proteins with established sedimentation coefficients were used: hemoglobin, alkaline phosphatase, catalase (Xu *et al.*, 2003). As an additional control, Rho hexamer was resolved on a separate glycerol gradient. First, each gradient was fractionated into 200 μL aliquots and the fraction numbers for the proteins with the established sedimentation coefficients were determined by absorbance measurements. They were plotted in relation to their sedimentation coefficients (Fig. 6a). Omp85 fractions were acetone precipitated and resolved on 10% SDS-PAGE. As shown in Fig. 6b, most of Omp85 was in fraction 23 and a small fraction of Omp85 was in fraction 9. Although the established sedimentation coefficient of Rho is 10.4 S (Geiselmann *et al.*, 1992), the glycerol gradients have consistently given 12 S based on these standards (Xu *et al.*, 2003). Using graph in Fig. 6a, the sedimentation coefficient of Rho 282 kDa hexamer was 12 S. A small fraction of Omp85 was in fraction 9 and had a sedimentation coefficient of 5.25 S. 5.25 S Omp85 was likely to correspond to an Omp85 monomer. Most of Omp85 was in fraction 23 and exhibited sedimentation coefficient of 14.5 S, greater than that of 282 kDa Rho hexamer. This fact pointed out that the majority of purified Omp85 formed tetramers with calculated molecular weight of about 356 kDa.

Quaternary structure of Omp85 *in vivo*: To determine the quaternary structure of Omp85 *in vivo,* the purified outer membranes from BL21/pET21B-Omp85 were resolved on semi-native LDS-PAGE. The whole electrophoresis was performed at 4°C at 1mAmp to prevent the oligomer disintegration or the protein denaturation. To enhance antibody recognition after electrophoresis

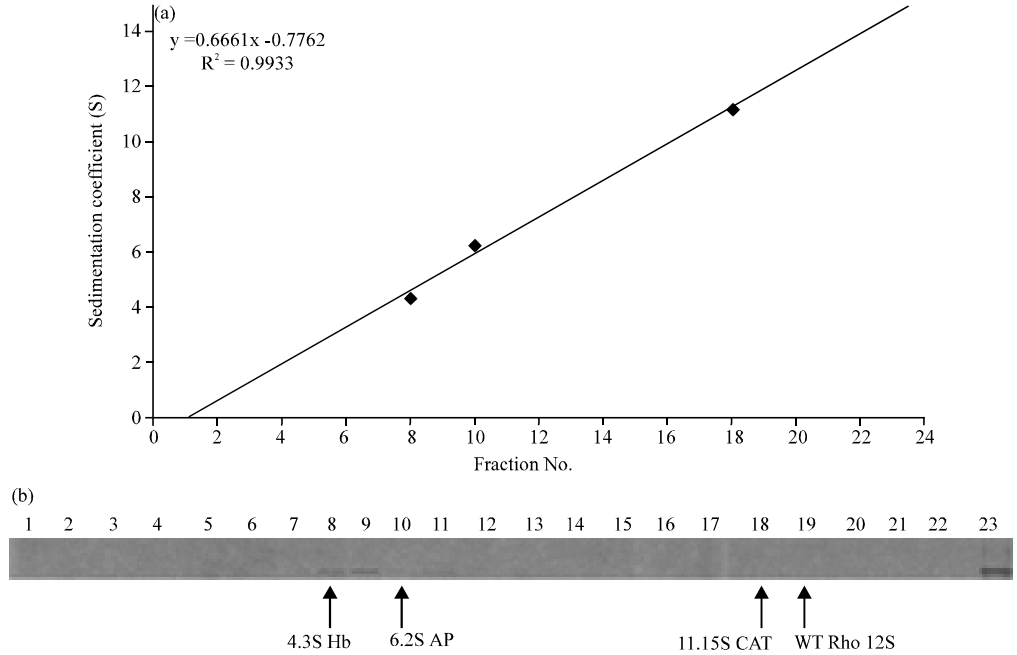

Fig. 6: Sedimentation studies of Omp85 show that Om85 exists mostly as a tetramer *in vitro*

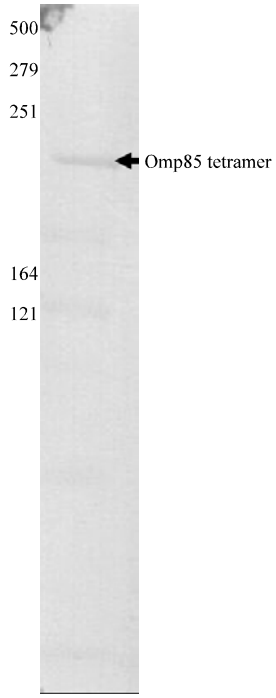

Fig. 7: Omp85 forms tetramers in the bacterial outer membrane

was complete, proteins on the gel were denatured by heating and transferred to nitrocellulose filter(Brok *et al.*, 1995). Since the Omp85 C-terminal had a His_6 tag, it was possible to distinguish it from other proteins in the outer membrane using anti-His monoclonal antibodies (Fig. 7).

The calculated molecular weight of the Omp85 tetramer was 354 kDa (89 kDa*4) and that of the heat-modified Omp85 tetramer was 199.36 kDa (354 kDa*0.56=199.36 kDa). There was a protein band at about 200 kDa. This band likely corresponded to the heat-modified Omp85 tetramer. *In vivo,* formation of Omp85 tetramers agreed the most with the sedimentation studies of Omp85, where most of the protein was also shown to form tetramers (Fig. 6).

DISCUSSION

Y. pestis causes plague in humans, rodents and fleas. Over the past years, the number of human cases has been increasing and *Y. pestis* strains resistant to multiple antibiotics have been isolated (Galimand *et al.*, 1997). The absence of adequate vaccine and the presence of antibiotic resistant strains highlight the urgent need for new antimicrobial drugs and vaccines against *Y. pestis*.

The subject of our study was Omp85 of *Y. pestis*. This outer membrane protein contains an integral membrane domain partially exposed to the extracellular space, thus accessible to antibodies and drugs. Omp85 belongs to a family of conserved proteins essential for cell viability and secretion of proteins, such as virulence factors, into the extracellular space. It has been shown to share homology to several proteins which serve as protective antigens.

Omp85 forms a complex with several proteins; however, it is the only protein in the complex that spans the whole outer membrane. Omp85 exists as a two-domain protein: the periplasmic N-terminal domain and the transmembrane ß-barrel C-terminal domain.

Our objective was to elucidate the quaternary structure of Omp85 of *Y. pestis in vitro* and *in vivo*. We cloned the *omp85* gene of *Y. pestis* into the pET21B plasmid of *E. coli*. We were able to express Omp85 protein and localize it to the outer bacterial membrane. We extracted the natively-folded full-length Omp85 from the bacterial outer membranes and purified it. Trypsin treatment and heat modifiability verified native folding of the purified Omp85. We assessed the quaternary structure of Omp85 using cross-linking with DSS and showed that Omp85 formed monomers, dimers and tetramers *in vitro*. Also, we determined the quaternary structure of Omp85 by determining its sedimentation coefficient and showed that Omp85 formed mostly tetramers *in vitro*. After this, we evaluated the quaternary structure of Omp85 using an anti-His_6 tag Western blot of the semi-native LDS-PAGE of the bacterial outer membranes and showed that Omp85 formed tetramers *in vivo*.

Different observations of oligomeric state of Omp85 family members under different conditions further highlights the dynamic nature of Omp85 and the multi-protein complex it forms. Our data agree with the crystal structure studies of FhaC that the purified Omp85 forms monomers and dimers (Clantin *et al.*, 2007). Although the knowledge gained from FhaC crystal structure is invaluable and enables structure predictions of Omp85, there are several important differences between FhaC and Omp85. First is that FhaC is not essential for bacteria viability and is not known to to be a part of the multi-protein complex, whereas Omp85 is. Furthermore, FhaC handles only one kind of substrate, whereas Om85 protein machinery is more versatile and handles substrates of a variety of sizes (Jacob-Dubuisson *et al.*, 2009).

In some of our experiments we were able to detect *in vitro* dimers and tetramers. Furthermore, our *in vivo* studies suggest that Omp85 exists as a tetramer. These results contradict the crystal and NMR structure studies, which only looked at Omp85 periplasmic domain or even shorter fragments. The discrepancy might me due to the fact that a contribution of transmembrane domain was overlooked in the earlier studies.

Our work agrees with Robert *et al.* (2006) that Omp85 family proteins are likely to form tetramers *in vitro* and *in vivo* but in addition monomer and dimer forms were also detected. It demonstrated that Omp85 mostly exists as a tetramer in the multi-protein apparatus that mediates the folding and incorporation of proteins into the bacterial outer membrane. Taken together, our data point out that Omp85 adopts a dynamic structure in order to facilitate ologomerization and insertion of outer membrane proteins into the membrane. It is interesting to note that the Omp85 tetrameric assembly does not seem to be very stable and it exits in equilibrium with the Omp85 monomers and dimers. This might suggest that the Omp85 tetramers are a part of the dynamic machinery involved in the folding and insertion of the outer membrane ß-barrel proteins into the bacterial outer membrane.

Although no model to date was able to account or explain all available data (Jacob-Dubuisson *et al.*, 2009; Knowles *et al.*, 2009), it is conceivable that transmembrane ß-barrels of four Omp85 come together and form a gated shielded central pore. The periplasmic domains of Omp85 might associate with the incoming substrate outer membrane proteins and guide them to the Omp85 central pore and mediate opening of the pore for the substrates to enter. In this shielded environment, the substrate outer membrane proteins fold into the characteristic ß-barrel structures and in the case of the trimeric outer membrane proteins, such as LamB, form oligomers. Once the folding and oligomerization are complete, Omp85 tetramer assembly might dis-associate into monomers to open laterally and release the outer membrane protein into the outer membrane.

In tetrameric Omp85, 20 periplasmic POTRA domains can come together to facilitate the folding and insertion of large substrates (Jacob-Dubuisson *et al.*, 2009). Also, they can speed up the processing of smaller more abundant proteins as nedded in response to changing environment.

Efforts of many groups are directed to determine the structure of either transmembrane or the periplasmic domain of Omp85 and its family members (Clantin *et al.*, 2007; Gatzeva-Topalova *et al.*, 2008; Knowles *et al.*, 2008). Other groups are focused on understanding multi-protein machinery, which includes Omp85 (Hagan *et al.*, 2010).

Our study is unique in that it presents the quaternary structure of non-denatured Omp85 as a whole protein with both its transmembrane and periplasmic domains intact, but without associating proteins.

The outcomes of our research provide the functional and structural characterization of different classes of bacterial outer membrane proteins. The applied relevance of this work is related to their potential use in the development of new vaccines and antimicrobials, potent weapons for fight against infectious diseases.

ACKNOWLEDGMENTS

I would like to thank Dr. Anne Delcour for kindly providing purified OmpU protein, Dr. Christos Stathopoulos and Dr. William Widger for their support and advice.

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