Published in final edited form as:

FEBS J. 2010 November ; 277(21): 4427–4437.

Resolving the native conformation of *Escherichia coli* OmpA

Alexander Negoda, Elena Negoda, and Rosetta N. Reusch

Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824, USA

Summary

The native conformation of the 325 residue outer membrane protein A (OmpA) of *Escherichia coli* has been a matter of contention. A narrow pore, two-domain structure has vied with a large pore, single-domain structure. Our recent studies show that serines S163 and S167 of the N-terminal domain (1–170) are modified in the cytoplasm by covalent attachment of oligo-(R)-3-hydroxybutyrates (cOHB), and further show that these modifications are essential for the N-terminal domain to incorporate into planar lipid bilayers as narrow pores (~80 pS, 1 M KCl, 22 °C). Here we examine the potential effect(s) of periplasmic modifications on pore structure by comparing OmpA isolated from outer membranes (M-OmpA) with OmpA isolated from cytoplasmic inclusion bodies (I-OmpA). Chemical and Western blot analysis and ¹H-NMR show that segment 264–325 in M-OmpA, but not in I-OmpA, is modified by cOHB. Moreover, a disulfide bond is formed between C₂₉₀ and C₃₀₂ by the periplasmic enzyme *DsbA*. Planar lipid bilayer studies indicate that narrow pores formed by M-OmpA undergo a temperature-induced transition into stable large pores (~450 pS, 1 M KCl, 22 °C) (E_a = 33.2 kcal/mol), but this transition does not occur with I-OmpA or with M-OmpA that has been exposed to disulfide bond reducing agents. The results suggest that the narrow pore is a folding intermediate, and demonstrate the decisive roles of cOHB-modification, disulfide bond formation and temperature in folding OmpA into its native large pore configuration.

Keywords

outer membrane protein; membrane protein; protein targeting; protein folding; oligo-(R)-3-hydroxybutyrates; disulfide bond; cOHB-modification

Introduction

OmpA, a major outer membrane protein of *Escherichia coli*, is a highly conserved and multifunctional integral membrane protein which has served as a model system for studies of outer-membrane targeting and protein folding [1]. However, despite intense study for several decades, the native structure of the protein has not yet been resolved.

A number of genetic and biochemical studies have provided evidence for a two-domain structure of OmpA in which the N-terminal domain (residues 1 to 170) crosses the membrane eight times in antiparallel β -strands, while the 155 residue C-terminal domain resides in the periplasm where it may interact with peptidoglycan [2–6]. Additional evidence for a two-domain structure comes from Raman spectroscopy [7] circular dichroism and fluorescence studies [8–16]. The crystal structure of the N-terminal 171 residues of OmpA, determined by Pautsch & Schulz [17,18], shows an eight-stranded amphipathic β -barrel with

no continuous water channel. High-resolution nuclear magnetic resonance (NMR) [19,20] and molecular dynamics studies [21,22] reveal some flexibility along the axis of the barrel, which could explain the formation of narrow ion-permeable pores in lipid bilayers [23]. It has also been suggested that a membrane-traversing narrow channel could be formed by repositioning a salt bridge in the pore interior [24].

However, there are also strong indications of a large pore conformation, consistent with the role of OmpA as a bacteriophage receptor [25–28] and participant in F-factor-dependent conjugation [29–31]. These physiological functions imply that it forms a pore large enough to allow passage of ssDNA. Stathopoulos [32] proposed that a large pore, 16-stranded β -barrel structure could be created by formation of eight additional β -strands from the C-terminal domain. A large pore conformation is also supported by studies of Sugawara and Nikaido [33] which showed that 2–3% of OmpA forms nonspecific diffusion channels in liposomes with an estimated pore size of ~ 1 nm. A large-pore conformer is further supported by single-channel conductance studies in planar lipid bilayers by Arora *et al.* [34], who found that OmpA formed channels with two distinct but interconvertible conductance states, one of 50–80 pS and a second of 260–320 pS, corresponding to a narrow and large channel, respectively. Full-length OmpA was required to observe both narrow and large channels; a truncate containing just the 170 residues of the N-terminal domain gave rise only to the narrow channels, indicating that the C-terminal portion takes part in formation of the large channels.

Membrane association and insertion of OmpA was shown by Kleinschmidt and Tamm [12] to be a multi-step process involving several partially folded intermediates. Significantly, the last step was observed only above room temperatures. Studies in our laboratory emphasize the importance of temperature in formation of the large pore conformer. Zakharian and Reusch [35,36] found that OmpA, isolated from outer membranes, forms narrow low-conductance pores in planar lipid bilayers (60–80 pS) at room temperature which undergo a temperature-induced transition to large pores (450 ± 60 pS). The transition of a single molecule of OmpA in the bilayer required ~ 2 days at 26 °C, ~ 2 hrs at 30 °C, ~ 30 min. at 37 °C and ~ 10 min. at 42 °C ($E_a = 33.2$ kcal/mol).

Recent studies in our laboratory introduce an additional factor in OmpA targeting and folding; namely, modification of the protein by covalent attachment of oligo-(R)-3-hydroxybutyrate (cOHB) [37]. OHB are flexible, amphiphilic, water-insoluble polyesters [38] which increase the hydrophobicity of polypeptide segments and thereby may facilitate their incorporation into bilayers. Studies by Bremer *et al.* [39], Klose *et al.* [40,41], and Freudl *et al.* [42] identified the segment 163–170 as essential for outer membrane integration. All proteins missing this fragment, known as the sorting signal, remain in the periplasm. Our studies showed that serines S163 and S167 of the sorting signal of OmpA are modified by cOHB [42]. The importance of these modifications was illustrated in subsequent studies which show that OmpA mutants lacking cOHB on S163 and S167 are incapable of incorporating into planar lipid bilayers [43].

Since the sorting signal is modified by cOHB in OmpA isolated from cytoplasmic inclusion bodies (I-Omp) or from outer membranes (M-OmpA), this modification occurs in the cytoplasm. Outer membrane proteins may undergo additional modification(s) in the periplasm. Here we compare I-OmpA and M-OmpA to investigate the potential effect(s) of periplasmic modifications on pore structure. In view of the high OHB polymerase activity in the periplasm [44], we explore the possibility of cOHB-modification(s) of the hydrophilic C-terminal domain. In addition, we examine the effect of the disulfide bond formed between residues 290 and 302 by the periplasmic enzyme DsbA [45–47].

Results

Pore conformations of M-OmpA and I-OmpA in planar lipid bilayers as a function of temperature

To determine whether OmpA undergoes modification(s) in the periplasm which influence the temperature-induced narrow to large pore transition, we compared the conductance of OmpA isolated from outer membranes (M-OmpA) with that of OmpA isolated from cytoplasmic inclusion bodies (I-OmpA) as a function of temperature. Both proteins were purified using LDS, incorporated into C₈E₄ micelles and then into planar bilayers of DPhPC between aqueous solutions of 1M KCl, 20 mM Hepes, pH 7.4 at 22 °C (see Methods). Both M-OmpA and I-OmpA formed narrow pores with a major conductance of ~80 pS at room temperature with long open times (>0.95); representative traces are shown in Figure 1A. Both channels display infrequent brief closures and occasional larger and smaller conductances which may be attributed to movements of the extra-bilayer loops and C-terminal segment of the protein into and out of the channel opening or to encounters with impermeant molecules. The micellar solutions of M-OmpA and I-OmpA were then each incubated at 40° C for 2 hrs, cooled to room temperature, and examined in planar bilayers as above at 22 °C. In agreement with our earlier findings [36], and as shown in Figure 1B, M-OmpA now formed large pores with a major conductance of ~ 450 pS and long open time (>0.98). I-OmpA, however, continued to form only narrow pores. I-OmpA persisted in forming only narrow pores, even after incubation at 42° C overnight. This difference between M-OmpA and I-OmpA after heating was confirmed by multiple observations of multiple preparations of each protein (see Methods). These studies indicate significant differences between M-OmpA and I-OmpA structure and infer that critical modification(s) of OmpA occur in the periplasm.

The effect of cOHB-modification of the C-terminal domain in the periplasm on the transition to the large pore conformation

In order for the large pore to form, a substantial portion of the hydrophilic C-terminal domain of OmpA (residues 171–325) must insert into the bilayer. Since cOHB-modification of S163 and S167 allowed the N-terminal domain to incorporate into planar lipid bilayers as narrow pores [43], it was considered that cOHB-modification of the C-terminal domain in the periplasm would increase the hydrophobicity of hydrophilic segments in this domain and thereby enable them to incorporate into the bilayer. In support of this premise, the periplasm of *E. coli* contains ~75% of total cellular cOHB polymerase activity [44].

Accordingly, we examined the C-terminal domains of M-OmpA and I-OmpA for the presence of cOHB. A large segment of the C-terminal domain can be obtained by digestion with the proteolytic enzyme chymotrypsin. This enzyme cuts after aromatic residues and there are no aromatic residues in the terminal 62 residues. Consequently, complete digestion of OmpA with chymotrypsin is expected to yield 29 small fragments (≤ 2.6 kDa) and one 6.6 kDa fragment containing the C-terminal residues 264–325. After extended digestion of M-OmpA and I-OmpA with a high ratio of protein to enzyme (20:1), SDS-PAGE of the digestion fragments of M-OmpA and I-OmpA displayed a band at MW ~ 7 kDa (Fig. 2A, lanes 1,2), identified by N-terminal sequencing as fragment 264–325 (MW 6.6 kDa). A Western blot of a similar gel probed with anti-OHB IgG indicated that this polypeptide in M-OmpA, but not in I-OmpA, was modified by cOHB (Fig. 2A, lanes 3,4).

The effect of cOHB-modification on the hydrophobicity of C-terminal segment 264–325 was next investigated by assessing the chloroform solubility of the polypeptides derived from M-OmpA and I-OmpA. Since OHB are chloroform-soluble, cOHB-containing polypeptides with a high ratio of OHB to protein may also be chloroform-soluble.

Accordingly, the solutions of chymotrypsin digests of M-OmpA and I-OmpA were each extracted with chloroform. Chemical assay (see Methods) of an aliquot of the chloroform solutions indicated ~4x more cOHB in the M-OmpA sample as in the I-OmpA sample. This assay confirms the presence of cOHB and evaluates the relative amounts of cOHB in the two samples but does not precisely quantitate the total amounts of cOHB since there are no cOHB standards. The presence of OHB in the chloroform extract of M-OmpA was confirmed by $^1\text{H-NMR}$. The $^1\text{H-NMR}$ spectrum (Fig. 3) includes resonances with the characteristic chemical shifts and coupling constants of the methylene and methine protons of OHB [58]; the methyl residues were obscured by other signals. The amount of cOHB in I-OmpA was insufficient for $^1\text{H-NMR}$ analysis.

The chloroform solutions were each evaporated into 2% sodium lauryl sulfate (SDS). The chloroform-soluble polypeptides were separated on 16.5% SDS-PAGE gels, and transferred to PVDF membrane. Ponceau S stain shows that the polypeptide, identified as 264–325 by N-terminal sequencing, is present in M-OmpA sample but not in the I-OmpA sample (Fig. 2B, lanes 1,2). A Western blot showed a strong positive reaction to anti-OHB IgG at ~7 kDa for the M-OmpA polypeptide; no reaction to the antibody was observed or expected for the I-OmpA polypeptide (Fig. 2B, lanes 3,4). There likely were an indeterminate number of cOHB-peptides in the chloroform extracts which were too small to be retained on 16.5% gels. The results indicate that segment 264–325 of M-OmpA is considerably more hydrophobic than the same segment of I-OmpA, and consequently more likely to insert into lipid bilayers.

The effect of the C₂₉₀–C₃₀₂ disulfide bond on the transition of OmpA to the large pore conformation

M-OmpA also differs from I-OmpA in that M-OmpA contains a disulfide bond which is formed between C₂₉₀ and C₃₀₂ in the periplasm by the oxidizing protein DsbA [45,46]. The importance of this disulfide bond to the narrow-large pore transition was next examined. When disulfide bond reducing agent 2-mercaptoethanol (2-ME) (Fig. 4A) or dithiothreitol (DTT) (1 mM) (Fig. 4B) was added to M-OmpA, either before or after its reconstitution into C₈E₄ micelles at room temperature, the protein formed narrow pores in planar bilayers which did not transform into large pores even after extended incubation at 40 °C. This result was confirmed by multiple observations of several preparations of M-OmpA (>2) treated with 2-ME and separately with DDT (see Methods). However, the addition of 2-ME or DTT to the protein *after* the large pore had been formed (by heating at 40 °C for 2 hr either in micelles or in the planar bilayer), did not disturb the large pore conformation (Fig. 4C). This result was confirmed by multiple observations of several preparations as described above. To test the stability of the large pore, up to 5 mM DTT was added to both sides of the bilayer with no discernible affect. These studies indicate that the disulfide bond is essential for the transition of the narrow pore to the large pore conformation, but it is not necessary for retention of the large pore conformation.

The effect of urea on OmpA pore structure conformation

In many studies of OmpA folding, OmpA is unfolded by treatment with urea under alkaline conditions at elevated temperatures in the presence of disulfide bond reducing agent 2-ME or DTT [8–10,12–16,24]. M-OmpA, purified in the presence of 8M urea and 0.05% 2-ME [24], forms narrow pores in DPhPC bilayers at 22 °C which display highly irregular conductance (65–100 pS) [43]. Here we isolated M-OmpA using the method of Kim et al. [16] which also employs both urea and 2-ME (see Methods). M-OmpA again formed irregular narrow pores of 60–90 pS at 22 °C. The M-OmpA was then heated to 40 °C, held at that temperature for 2 hrs and cooled to room temperature. The preparation still formed

only irregular narrow pores. Even after incubation o/n at 40 °C, the protein remained in the narrow pore conformation (Fig. 5, upper trace).

Since 2-ME, itself, prevents the formation of the large pore conformer, the urea was next individually examined for its influence on the narrow to large pore transition of OmpA. M-OmpA was again prepared by the method of Kim et al. [16] except that 2-ME was omitted. After reconstitution in C₈E₄ micelles, M-OmpA formed irregular narrow pores of 60–90 pS in planar lipid bilayers of DPhPC which transitioned after incubation at 40 °C for 2 hrs into irregular pores with a wide range of conductances extending from 180–380 pS at 22 °C (Fig. 5, lower trace). The current records resemble those of large pores observed by Arora et al. [34] with OmpA which was also prepared with urea but without 2-ME. They suggest that one or more segments of the C-terminal domain are attempting to insert into the bilayer but are unable to become part of a stable large pore structure. Further incubation at room temperature or at 40 °C o/n had no significant effect. M-OmpA was also prepared using LDS (see Methods), and then incubated at room temperature with 8M urea or alternatively 1M urea at pH 7.4 for ~ 2 hrs. The urea-exposed M-OmpA was subsequently diluted and reconstituted into C₈E₄ micelles, heated at 40 °C for 2 hrs and cooled to room temperature. In all cases, exposure to urea produced noisy pores with a wide range of conductances of intermediate magnitude (180–380 pS), i.e. higher than that of narrow pores but lower than that of large-pores obtained by purification using LDS (~450 pS) (Fig. 1, M-OmpA bottom trace). As above, these results were confirmed by multiple observations of several separate preparations of each protein (see Methods). The results indicate that urea does not prevent the narrow to large pore transition but it has a negative effect on pore structure.

Discussion

Our studies support the premise that native OmpA is a large pore with a conductance of ~ 450 pS in 1M KCl at 22°C. Previously we showed that S163 and S167 of the N-terminal domain are modified by cOHB in the cytoplasm [37]. Here we find that segment 264–325 of the C-terminal domain is modified by cOHB in the periplasm. Another periplasmic modification, namely C₂₉₀–C₃₀₂ disulfide bond formation by the enzyme DsbA, has been reported by Bardwell et al [45]. We find that all of these modifications and incubation at elevated temperatures (Ea=33.2 kcal/mol) [36], are decisive factors in folding OmpA into its large pore conformation.

In vivo, nascent OmpA is modified on S163 and S167 by cOHB, escorted across the plasma membrane by the Sec translocation system and deposited into the periplasm [48]. The N-terminal domain may then insert into the outer membrane bilayer as a narrow pore (Fig. 1), while the hydrophilic C-terminal domain remains in the periplasm. Enzymatic attachment of OHB to residues in this segment increases their hydrophobicity and thereby facilitates their insertion into the outer membrane bilayer at the physiological temperatures of *E. coli* (~37 °C). In this respect, Dai et al. [44] found OHB polymerase in both cytoplasmic and periplasmic fractions, but the majority of this activity (~ 75%) is in the periplasm. The enhanced hydrophobicity conferred by cOHB-modification is demonstrated by the chloroform solubility of polypeptide 264–325 from M-OmpA, but not from I-OmpA (Fig. 2B).

When OmpA is extracted from membranes using denaturing agents such as urea or ionic detergents, it initially adopts the narrow pore two-domain conformation. However, if heated in lipids, OmpA refolds into a large pore [34,36]. Zakharian and Reusch [36] showed that the large pore conformation, once formed, is very stable to temperature change - it is unaffected by cooling, even storage below freezing. However, large pores rapidly revert to narrow pores when exposed to urea or ionic detergents [36]. Significantly, the relatively

high E_a for the narrow-large pore transition means that it does not occur at an appreciable rate at room temperatures [36]. The low percentage of large pores detected in liposomes by Sugawara and Nikaido [42] can be attributed to their observations being made at room temperature.

Although modifications by cOHB and elevated temperatures are both essential to formation of the large pore conformer, they are not sufficient. While cOHB modification is an effective process for increasing the hydrophobicity of polypeptide segments destined to remain within the bilayer, it may not be suitable for those segments of the C-terminal domain that must traverse the bilayer to reach the extracellular aqueous medium. In the Stathopoulos model [32], the longest extracellular loop formed during the folding of the C-terminal domain consists of residues 288–307. This segment includes the two cysteines residues as well as six charged residues (3 positive and 3 negative). Molecular modeling studies suggest that formation of a C₂₉₀–C₃₀₂ disulfide bond may facilitate bilayer transfer of this putative segment by packaging it into a more compact structure and enabling the formation of salt bridges between the oppositely charged residues (Fig. 6). This conjecture is in agreement with our planar bilayer studies which show that the C₂₉₀–C₃₀₂ disulfide bond is essential for the narrow to large pore transition but it is no longer essential once the large pore conformer has formed and this segment has reached the extracellular fluid (Fig. 4). It is noteworthy that disulfide bond reducing agents were not present in liposome studies by Sugawara and Nikaido [33] or in planar lipid bilayer studies by Arora et al. [34] in which the large pore conformer was observed, but were present in all of the folding studies which concluded that the narrow pore is the native structure [8–10,12–16,24].

Although urea will not prevent the formation of the large pore conformer, it is harmful to large pore structure. OmpA exposed to urea forms irregular pores with conductances that vary widely in magnitude. They undergo the temperature-induced narrow to large pore transition (Fig. 5, bottom trace) but they are never as high-conducting as pores formed when OmpA is purified with LDS (Fig. 1, M-OmpA bottom trace). The harmful effect of urea may be due to its propensity to form isocyanic acid on exposure to heat and alkali, resulting in carbamylation of lysines [49]. Indeed, the irregular conductance of the large pores observed by Arora et al. [34] can be attributed to the use of urea in isolation and purification procedures.

An additional impediment to resolving the native structure of OmpA has been a misguided reliance on the electrophoretic mobility of OmpA on SDS-PAGE gels to recognize the native state [12–16,24]. OmpA is heat-modifiable [50]. When the protein is boiled in sodium dodecyl sulfate (SDS) before SDS-PAGE, it migrates at 35 kDa but when unheated it migrates at 30 kDa. The 35 kDa protein has been considered to be the unfolded form and the 30 kDa protein the native form. However, Zakharian and Reusch [36] showed that both narrow pore and large pore conformers migrate at 30 kDa; only completely unfolded OmpA migrates at 35 kDa. Accordingly one cannot distinguish narrow and large pore conformers by electrophoretic migration.

In summary, our studies show that native OmpA is a large pore (possibly 16 beta barrels), consistent with its physiological functions. They also identify several factors that inhibit or prevent the refolding of the narrow pore intermediate into the large pore conformation and they distinguish two important physiological strategies used to facilitate OmpA targeting and folding - cOHB modification and disulfide bond formation. The former may be used to incorporate hydrophilic polypeptide segments within the bilayer and the latter to facilitate the translocation of long hydrophilic segments across the bilayer into the extracellular aqueous medium. Moreover, the presence of strong OHB polymerase activity [44] and enzymatic systems for disulfide bond formation in the periplasm [45–47] suggest that

cOHB-modification and disulfide bond formation may be important general mechanisms in the targeting and folding of outer membrane proteins.

Materials and Methods

Purification of OmpA from outer membranes (M-OmpA)

OmpA was extracted from the outer membranes of *E. coli* JM109 by a modification of the method of Sugawara and Nikaido [23]. Early stationary-phase cells were suspended in 20 mM trishydroxymethyl aminomethane chloride (Tris-Cl), pH 7.5, 5 mM ethylenediamine tetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF) and disintegrated by ultrasonication (Branson, Danbury CT USA). Unbroken cells were removed by centrifugation at 4000 rpm for 10 min (Beckman GSA rotor, Brea CA USA) at 4 °C and crude outer membrane fractions were recovered by centrifugation at 12,000 rpm for 30 min (Beckman SS 34 rotor, Brea CA USA) at 4 °C. Outer membranes were suspended in 0.3% lithium dodecyl sulfate (LDS) containing 5 mM EDTA and 20 mM Hepes, pH 7.5, to a final protein concentration of 2 mg/ml. After 1 hr on a shaker at 4°C, the suspension was centrifuged at 35,000 rpm for 45 min (Beckman Type 50 rotor, Brea CA USA). The supernatant was discarded and the pellet was resuspended in 2% LDS, 5 mM EDTA, 20 mM KHepes, pH 7.5, and gently mixed at 4 °C for >1 hr. The suspension was then again centrifuged at 35,000 rpm in the same rotor for 45 min. The pellet was discarded and the supernatant, containing soluble OmpA, was loaded onto a column of Sephacryl S-300 1.6 ×60 cm, HiPrep, (GE Healthcare, Piscataway NJ USA) which had been equilibrated with 0.05% LDS, 0.4 M LiCl, 20 mM KHepes, pH 7.5. Fractions were eluted with the same solvent and examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). OmpA-rich fractions were combined, concentrated using Amicon Centricon-10 Filter units (Millipore, Billerica MA USA). For further purification, samples were loaded onto a column of Superdex 75 10/300; HiPrep, (GE Healthcare, Piscataway NJ USA) equilibrated with the same solvent.

M-OmpA was also isolated from outer membranes of *E. coli* JM109 with urea essentially as described by Kim et al. [16]. Briefly, cells were suspended in a solution of sucrose (0.75M), 10 mM Tris-Cl (pH 7.8), 20 mM EDTA. Lysozyme was added (0.5 mg/ml) and cells were sonicated on ice for 5 min. Unbroken cells were removed by low speed centrifugation (1500 x g; 15 min.) and outer membranes were pelleted by centrifugation at 25,000 x g for 20 min (Beckman Type 50 rotor, Brea CA USA). The pellet was resuspended in 3.5 M urea, 20 mM Tris-Cl, pH 9.0, 0.05% 2-ME by stirring in a 50 °C water bath. The solution was centrifuged at 100,000 x g for 90 min. in the same rotor and the pellet was resuspended in 1:1 mixture of isopropanol and a solution of 8M urea, 15 mM Tris-Cl, pH 8.5, 0.1% 2-ME, stirred at 50 °C for 30 min and centrifuged at 100,000 x g for 90 min. The supernatant containing extracted OmpA was then purified by size-exclusion chromatography as described above.

Purification of OmpA from inclusion bodies (I-OmpA)

Mature OmpA was overexpressed in *E. coli* BL21(DE3)pLysS cells (Novagen EMD, Gibbstown NJ USA) containing the pET(-45b+)-His-ompA plasmid, and was grown in LB medium supplemented with 50 µg/ml ampicillin and 30 µg/ml chloramphenicol at 37 °C with aeration to an A₆₀₀ of 0.4. Protein expression was induced by the addition of 0.2 mM isopropyl-1-thio-3-D-galactopyranoside (IPTG), and the cells were allowed to grow at 37 °C for an additional 2–3h before harvesting by centrifugation. Cells were disintegrated by ultrasonication as above and inclusion bodies were collected by centrifugation at 15,000 rpm for 30 min (Beckman SS 34 rotor, Brea CA USA). His-OmpA was extracted and purified by Ni-agarose chromatography as described by the manufacturer (Qiagen). Alternatively, the His-OmpA was extracted with LDS and purified by chromatography on a Sephacryl S-300

1.6 ×60 cm column, HiPrep, (GE Healthcare, Piscataway NJ USA) using the same methods described for outer membranes above.

Planar lipid bilayer studies

M-OmpA and I-OmpA preparations were concentrated to ~ 1 mg/ml by centrifugal filtration using 10K Centricon filters. Buffer substitution was then performed 5X with 20 mM n-octyl tetraethylene glycol monoether (C₈E₄) in 20 mM KHepes, pH 7.4 using the same filters. The concentrate was then diluted with the C₈E₄ solution to 0.1 mg/ml. This solution (1 µl) was added to the *cis* side of a planar bilayer formed with synthetic diphtanoylphosphatidylcholine (DPhPC) (Avanti Polar lipids). Planar lipid bilayers were formed from a solution of DPhPC in n-decane (Sigma-Aldrich, Union City CA USA) at a concentration of ~ 17 mg/ml. The solution was used to paint a bilayer in an aperture of ~150 µm diameter between aqueous solutions of 1M KCl in 20 mM Hepes, pH 7.4 in a Delrin cup (Warner Instruments, Hamden CT USA). All salts were ultrapure (Sigma-Aldrich, St. Louis MO USA). After the bilayer was formed, a solution of OmpA in C₈E₄ (1 µl of 0.1 mg/ml) was added to the *cis* compartment.

Unitary currents were recorded with an integrating patch clamp amplifier (Axopatch 200A, Axon Instr., Union City CA USA). The *trans* solution (voltage command side) was connected to a CV 201A head stage input and the *cis* solution was held at virtual ground via a pair of matched Ag-AgCl electrodes. Currents through the voltage-clamped bilayers were low-pass filtered at 10 kHz and recorded after digitization through an analog to digital converter Digidata 1322A, (Axon Instr., Union City CA USA). Data were filtered through an 8 pole Bessel filter 9021 PF, (Frequency Devices, Ottawa IL USA) and digitized at 1 kHz using pClamp 9.0 software (Axon Instr., Union City CA USA). Single-channel conductance events were identified and analyzed by using Clampfit9 software (Axon Instr., Union City CA USA). The data were averaged from > 10 independent recordings. Each recording was 2 to 10 minutes long. Traces shown are representative of records from at least 10 separate observations of each of 2–5 separate preparations.

Digestion of OmpA with chymotrypsin

M-OmpA and I-OmpA (~500 µg) were each dissolved in 0.1% RapiGest SF and bovine chymotrypsin (sequencing grade), modified to inhibit trace trypsin activity and reduce autolysis (Princeton Separations, Adelphia, N.J., USA), was added to each (protein:enzyme; 20:1). The solutions were incubated at 30 °C for 4 hrs and then overnight at room temperature. A portion of the digests was set aside for SDS-PAGE, Western blot analysis and N-terminal sequencing and the remainder was extracted with chloroform (3x). The chloroform solutions were combined and back-extracted 1x with water. A small volume (~ 50µl) of 2% sodium dodecyl sulfate was added and the chloroform was evaporated with a stream of dry nitrogen gas.

SDS-PAGE and Western blot

Laemmli loading buffer containing 2% β-mercaptoethanol was added to each chymotrypsin digest sample (original and chloroform-soluble) and each was separated by electrophoresis on 16.5% SDS-PAGE gels. The gels were transferred to supported nitrocellulose or PVDF membrane (sequencing grade) (Bio-Rad, Hercules, CA, USA) in 25mM Tris-Glycine buffer, pH 8.3, using a Mini Trans-Blot electrophoretic cell (Bio-Rad, Hercules, CA, USA). To test for protein, the membrane was stained with 0.1% Ponceau S in 1% acetic acid and destained with 5% acetic acid. For Western blot, the membranes were blocked with 1.25% electrophoresis grade gelatin (Bio-Rad, Hercules, CA, USA) in Tris-buffered saline, pH 7.5, 0.1% Tween-20. Primary incubation was with polyclonal anti-OHB IgG in blocking buffer. The antibody was produced in rabbits to a synthetic 8mer of OHB (courtesy of D. Seebach,

ETH Zürich) conjugated to electrophoresis-pure gelatin (Bio-Rad, Hercules, CA, USA) by Metabolix Inc. (Cambridge, MA, USA) and purified by protein A chromatography (Invitrogen, Carlsbad CA USA). The second antibody was goat anti-rabbit alkaline phosphatase conjugate (Bio-Rad, Hercules, CA, USA) in the same buffer. Color development was performed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) (Bio-Rad, Hercules, CA, USA). Standards were Kaleidoscope peptides (Bio-Rad, Hercules, CA, USA).

Chemical assay for cOHB

The procedure used is an adaptation of the method of Karr et al. [62] as described by Huang and Reusch [57]. Chloroform was evaporated, concentrated sulfuric acid (0.6 ml) was added to the dried sample and the mixture was heated in a dry heating block (Thermo Scientific, Rockford IL USA) at 120 °C for 20 min. The tube was cooled on ice, 1.2 ml of saturated sodium sulfate was added, and the solution was extracted 3x with 2 ml of dichloromethane. Sodium hydroxide (100 µl of 5N) was added to the extract to convert volatile crotonic acid to crotonate, and the dichloromethane was evaporated with a stream of nitrogen. The residue was acidified by the addition of 5N sulfuric acid and filtered with a 0.45 mm PVDF syringe filter (Whatman). The filtrate was chromatographed on an HPLC Aminex HPX-87H ion exclusion organic acid analysis column (Bio-Rad, Hercules, CA, USA) with 0.014 N H₂SO₄ as eluant at a flow rate of 0.6 ml per min. The crotonic acid peak was identified by comparison of elution time with that of known crotonic acid and by its UV absorption spectrum. Crotonic acid content was estimated by peak area using granule PHB (Sigma-Aldrich, Union City, CA, USA) as standards.

¹H-NMR Spectroscopy

For ¹H-NMR spectroscopy, ~15 mg of M-OmpA were digested with chymotrypsin as above. The digests were extracted with chloroform (3x) and chloroform was evaporated. The residue was treated with 5% sodium hypochlorite solution to degrade protein (cOHB is more resistant to alkaline hydrolysis than free OHB [63]). Chloroform was again added, and after thorough mixing the aqueous hypochlorite layer was removed. This process was repeated 5x. The final chloroform solution was washed (3x) with distilled water and the chloroform was then evaporated. The residue was dissolved in 250 µl deuterated chloroform in a Shigemi thin wall NMR sample tube (Shigemi Inc., Allison Park PA USA) and examined in a Varian Inova-600 MHz superconducting NMR spectrometer (Palo Alto CA USA) at 25 °C.

Molecular Modeling

The molecular model of residues 288–307 was created and minimized by molecular mechanics using Hyper-Chem 5.0 (Hypercube Inc. Gainesville FL USA).

Acknowledgments

We thank William H. Reusch for the molecular modeling of the C-terminal segment of OmpA which contains a disulfide bond. This work was partially supported by NIH grant GM054090 and by a grant from Metabolix Inc., Cambridge, MA.

Abbreviations used

OmpA	outer membrane protein A
M-OmpA	OmpA isolated from outer membranes
I-OmpA	OmpA isolated from cytoplasmic inclusion bodies

OHB	oligo-(R)-3-hydroxybutyrate
cOHB	conjugated OHB
DPhPC	diphytanoylphosphatidylcholine
C8E4	tetraethylene glycol monoethyl ether
Tris	trishydroxymethylaminomethane
LDS	lithium dodecyl sulfate
2-ME	2-mercaptoethanol
DDT	dithiothreitol
PMSF	phenylmethylsulfonyl fluoride
Hepes	4-2-(hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG	isopropyl-1-thio-3-D-galactopyranoside
PVDF	polyvinylidene fluoride
LDS	lithium dodecyl sulfate

References

1. Smith SG, Mahon V, Lambert MA, Fagan RP. A molecular Swiss army knife: OmpA structure, function and expression. *FEMS Microbiol Lett.* 2007; 273:1–11. [PubMed: 17559395]
2. Klose M, Storiko A, Stierhof Y-D, Hindennach I, Mutschler B, Henning U. Membrane assembly of outer membrane protein OmpA of *E. coli*. *J Biol Chem.* 1993; 268:25664–25670. [PubMed: 8245002]
3. Ried G, Koebnik R, Hindennach I, Mutschler B, Henning U. Membrane topology and assembly of the outer membrane protein OmpA of *Escherichia coli* K12. *Mol Gen Genet.* 1994; 243:127–135. [PubMed: 8177210]
4. Koebnik R, Kramer L. Membrane assembly of circularly permuted variants of the *E. coli* outer membrane protein OmpA. *J Mol Biol.* 1995; 250:617–626. [PubMed: 7623380]
5. Koebnik R. Membrane assembly of the *Escherichia coli* outer membrane protein OmpA: exploring sequence constraints on transmembrane beta-strands. *J Mol Biol.* 1999; 285:1801–1810. [PubMed: 9917413]
6. Koebnik R, Locher KP, Van Gelder P. Structure and function of bacterial outer membrane proteins: barrels in a nutshell. *Mol Microbiol.* 2000; 37:239–253. [PubMed: 10931321]
7. Vogel H, Jähnig F. Models for the structure of outer membrane proteins of *Escherichia coli* derived from Raman spectroscopy and prediction methods. *J Mol Biol.* 1986; 190:191–199. [PubMed: 3025450]
8. Surrey T, Jähnig F. Refolding and oriented insertion of a membrane protein into a lipid bilayer. *Proc Natl Acad Sci USA.* 1992; 89:7457–7461. [PubMed: 1502158]
9. Surrey T, Jähnig F. Kinetics of folding and membrane insertion of a β -barrel membrane protein. *J Biol Chem.* 1995; 270:28199–28203. [PubMed: 7499313]
10. Rodionova NA, Tatulian SA, Surrey T, Jähnig F, Tamm LK. Characterization of two membrane-bound forms of OmpA. *Biochemistry.* 1995; 34:1921–1929. [PubMed: 7849052]
11. Sugawara E, Steiert M, Rouhani S, Nikaido H. Secondary structure of the outer membrane proteins OmpA of *Escherichia coli* and OprF of *Pseudomonas aeruginosa*. *J Bacteriol.* 1996; 178:6067–6069. [PubMed: 8830709]
12. Kleinschmidt JH, Tamm LK. Folding intermediates of a beta-barrel membrane protein. Kinetic evidence for a multistep membrane insertion mechanism. *Biochemistry.* 1996; 35:12993–13000. [PubMed: 8855933]

13. Kleinschmidt JH, Tamm LK. Time-resolved distance determination by tryptophan fluorescence quenching: probing intermediates in membrane protein folding. *Biochemistry*. 1999; 38:4996–5005. [PubMed: 10213602]
14. Kleinschmidt JH, Tamm LK. Outer membrane protein A of *Escherichia coli* inserts and folds into lipid bilayers by a concerted mechanism. *Biochemistry*. 1999; 38:5006–5016. [PubMed: 10213603]
15. Kleinschmidt JH, Wiener MC, Tamm LK. Outer membrane protein A of *E. coli* folds into detergent micelles, but not in the presence of monomeric detergent. *Prot Sci*. 1999; 8:2065–2071.
16. Kim JE, Arjara G, Richards JH, Gray HB, Winkler JR. Probing folded and unfolded states of outer membrane protein a with steady-state and time-resolved tryptophan fluorescence. *J Phys Chem B*. 2006; 110:17656–17662. [PubMed: 16942111]
17. Pautsch A, Schulz GE. Structure of the outer membrane protein A transmembrane domain. *Nat Struct Biol*. 1998; 5:1013–1017. [PubMed: 9808047]
18. Pautsch A, Schulz GE. High-resolution structure of the OmpA membrane domain. *J Mol Biol*. 2000; 298:273–282. [PubMed: 10764596]
19. Arora A, Abildgaard F, Bushweller JH, Tamm LK. Structure of outer membrane protein A transmembrane domain by NMR spectroscopy. *Nat Struct Biol*. 2001; 8:334–338. [PubMed: 11276254]
20. Johansson MU, Alioth S, Hu K, Walser R, Koebnik R, Pervushin K. A minimal transmembrane beta-barrel platform protein studied by nuclear magnetic resonance. *Biochemistry*. 2007; 46:1128–1140. [PubMed: 17260943]
21. Bond PJ, Faraldo-Gómez JD, Sansom MS. OmpA: a pore or not a pore? Simulation and modeling studies. *Biophys J*. 2002; 83:763–775. [PubMed: 12124263]
22. Cox K, Bond PJ, Grottesi A, Baaden M, Sansom MS. Outer membrane proteins: comparing X-ray and NMR structures by MD simulations in lipid bilayers. *Eur Biophys J*. 2008; 37:131–141. [PubMed: 17551722]
23. Sugawara E, Nikaïdo H. Pore-forming activity of OmpA protein of *Escherichia coli*. *J Biol Chem*. 1992; 267:2507–2511. [PubMed: 1370823]
24. Hong H, Szabo G, Tamm LK. Electrostatic couplings in OmpA ion-channel gating suggest a mechanism for pore opening. *Nat Chem Biol*. 2006; 2:627–635. [PubMed: 17041590]
25. Morona R, Klose M, Henning U. *Escherichia coli* K-12 outer membrane protein OmpA as a bacteriophage receptor: Analysis of mutant genes expressing altered proteins. *J Bacteriol*. 1984; 159:570–578. [PubMed: 6086577]
26. Morona R, Kramer C, Henning U. Bacteriophage receptor area of outer membrane protein OmpA of *Escherichia coli* K-12. *J Bacteriol*. 1985; 164:539–543. [PubMed: 3902787]
27. Koebnik R. Structural and functional roles of the surface exposed loops of the beta-barrel membrane protein OmpA from *Escherichia coli*. *J Bacteriol*. 1999; 181:3688–3694. [PubMed: 10368142]
28. Power ML, Ferrari BC, Littlefield-Wyer J, Gordon DM, Slade MB, Veal DA. A naturally occurring novel allele of *Escherichia coli* outer membrane protein A reduces sensitivity to bacteriophage. *Appl Environ Microbiol*. 2006; 72:7930–7932. [PubMed: 16980421]
29. Schweizer M, Henning U. Action of major outer cell envelope membrane protein in conjugation of *Escherichia coli* K-12. *J Bacteriol*. 1977; 129:1651–1652. [PubMed: 321438]
30. Klimke WA, Frost LS. Genetic analysis of the role of the transfer gene, traN, of the F and R100-1 plasmids in mating pair stabilization during conjugation. *J Bacteriol*. 1998; 180:4036–4043. [PubMed: 9696748]
31. Klimke WA, Rypien CD, Klinger B, Kennedy RA, Rodriguez-Maillard JM, Frost LS. The mating pair stabilization protein, TraN, of the F plasmid is an outer-membrane protein with two regions that are important for its function in conjugation. *Microbiol*. 2005; 151:3527–3540.
32. Stathopoulos C. An alternative topological model for *Escherichia coli* OmpA. *Protein Sci* 5, 170–173.41. Sugawara E & Nikaïdo H (1994) OmpA protein of *Escherichia coli* outer membrane occurs in open and closed channel forms. *J Biol Chem*. 1996; 269:17981–17988.
33. Sugawara E, Nikaïdo H. OmpA protein of *Escherichia coli* outer membrane occurs in open and closed channel forms. *J Biol Chem*. 1994; 269:17981–17988. [PubMed: 7517935]

34. Arora A, Rinehart D, Szabo G, Tamm LK. Refolded outer membrane protein A of *Escherichia coli* forms ion channels with two conductance states in planar lipid bilayers. *J Biol Chem.* 2000; 275:1594–1600. [PubMed: 10636850]
35. Zakharian E, Reusch RN. Outer membrane protein A of *Escherichia coli* forms temperature-sensitive channels in planar lipid bilayers. *FEBS Lett.* 2003; 555:229–235. [PubMed: 14644420]
36. Zakharian E, Reusch RN. Kinetics of folding of *Escherichia coli* OmpA from narrow to large pore conformation in a planar bilayer. *Biochemistry.* 2005; 44:6701–6707. [PubMed: 15850404]
37. Xian M, Fuerst MM, Shabalin Y, Reusch RN. Sorting signal of *Escherichia coli* OmpA is modified by oligo-(R)-3-hydroxybutyrate. *Biochim Biophys Acta.* 2007; 1768:2660–2666. [PubMed: 17659252]
38. Waser P, Rueping M, Seebach D, Duchardt E, Schwalbe H. On the solution structure of PHB: Preparation and NMR analysis of isotopically labeled oligo(R)-3-hydroxybutanoic acids (OHBs). *Helv Chim Acta.* 2010; 84:1821–1845.
39. Bremer E, Cole ST, Hindemach I, Henning U, Beck E, Kurz C, Schaller H. Export of a protein into the outer membrane of *Escherichia coli* K12. Stable incorporation of the OmpA protein requires less than 193 amino-terminal amino-acid residues. *Eur J Biochem.* 1982; 122:223–231. [PubMed: 7037401]
40. Klose M, Schwarz H, MacIntyre S, Freudl R, Eschbach M, Henning U. Internal deletions in the gene for an *Escherichia coli* outer membrane protein define an area possibly important for recognition of the outer membrane by this polypeptide. *J Biol Chem.* 1988a; 263:13291–13296. [PubMed: 3047120]
41. Klose M, MacIntyre S, Schwarz H, Henning U. The influence of amino substitutions within the mature part of an *Escherichia coli* outer membrane protein OmpA on assembly of the polypeptide into its membrane. *J Biol Chem.* 1988b; 263:13297–13302. [PubMed: 3047121]
42. Freudl R, Klose M, Henning U. Export and sorting of the *Escherichia coli* outer membrane protein OmpA. *J Bioenerg Biomembr.* 1990; 22:441–449. [PubMed: 2202726]
43. Negoda A, Negoda E, Reusch RN. Oligo-(R)-3-hydroxybutyrate modification of sorting signal enables pore formation by *E. coli* OmpA. *Biochim Biophys Acta.* 2010; 1798:1480–1484. [PubMed: 20004640]
44. Dai D, Reusch RN. Poly-3-hydroxybutyrate synthase from the periplasm of *Escherichia coli*. *Biochem Biophys Res Commun.* 2008; 374:485–489. [PubMed: 18640095]
45. Bardwell JC, McGovern K, Beckwith J. Identification of a protein required for disulfide bond formation *in vivo*. *Cell.* 1991; 67:581–589. [PubMed: 1934062]
46. Collet J-F, Bardwell JCA. Oxidative protein folding in bacteria. *Mol Microbiol.* 2002; 44:1–8. [PubMed: 11967064]
47. Ito K, Inaba K. The disulfide bond formation Dsb system. *Curr Opin Struct Biol.* 2008; 18:450–458. [PubMed: 18406599]
56. Reusch RN. Biological complexes of poly-beta-hydroxybutyrate. *FEMS Microbiol Revs.* 1992; 9:119–129. [PubMed: 1476771]
57. Huang R, Reusch RN. Poly-3-hydroxybutyrate is associated with specific proteins in the cytoplasm and membranes of *Escherichia coli*. *J Biol Chem.* 1996; 271:22196–22302. [PubMed: 8703033]
58. Seebach D, Brunner A, Bürger HM, Schneider J, Reusch RN. Isolation and ¹H-NMR spectroscopic identification of poly(3-hydroxybutanoate) from prokaryotic and eukaryotic organisms. *Eur J Biochem.* 1994; 224:317–328. [PubMed: 7925344]
59. Wickner W, Driessen AJM, Hartl F-U. The enzymology of protein translocation across the *Escherichia coli* plasma membrane. *Ann Rev.* 1991; 60:101–124.
60. Hagel P, Gerding JJT, Fieggen W, Bloemendal H. Cyanate formation in solutions of urea. *Biochim Biophys Acta.* 1971; 243:366–373. [PubMed: 5129584]
61. Schweizer M, Hindemach I, Garten W, Henning U. Major proteins of the *Escherichia coli* outer cell envelope membrane. Interaction of protein II with lipopolysaccharide. *Eur J Biochem.* 1978; 82:211–217. [PubMed: 340230]
62. Karr DB, Water JK, Emerich DW. Analysis of poly-beta-hydroxybutyrate in *Rhizobium japonicum* bacteroids by ion-exclusion high-pressure liquid chromatography and UV detection. *Appl Environ Microbiol.* 1983; 46:1339–1344. [PubMed: 16346443]

63. Reusch RN, Bryant EM. Enhanced hydrolytic stability of short-chain poly(R)-3- hydroxybutyrate conjugated to native *E. coli* cytoplasmic proteins. *Helv Chim Acta*. 2002; 85:3867–3871.

Figure 1. Representative single-channel current traces of OmpA isolated from outer membranes (M-OmpA) and from cytoplasmic inclusion bodies (I-OmpA)
 Each protein was isolated with LDS, reconstituted in C₈E₄ micelles and incorporated into bilayers of DPhPC between aqueous solutions of 20 mM Hepes, pH 7.4, 1 M KCl at 22 °C (see Methods). Upper traces (A) - M-OmpA and I-OmpA at 22 °C; lower traces (B) - M-OmpA and I-OmpA at 22 °C after incubation at 40 °C for 2 hrs. The closed state is indicated by the bar at the right of each trace. The clamping potential was +100 mV with respect to ground (*trans*). The corresponding histograms from one minute of continuous recording show the distribution of conductance magnitudes.

Figure 2.

A. cOHB-modification of OmpA segment 264–325. M, M-OmpA; I, I-OmpA. Lanes 1,2: SDS-PAGE (16.5%) of chymotrypsin digestion fragments: Lanes 3,4: Supported nitrocellulose blot of 16.5% SDS-PAGE gel probed with anti-OHB IgG.

B. Chloroform-solubility of OmpA segment 264–325. PVDF blot of SDS-PAGE (16.5%) of chloroform-soluble chymotrypsin digestion fragments. Lanes 1,2 - stained with Ponceau S. Lanes 3,4 - probed with anti-OHB IgG.

Figure 3. ^1H -NMR spectrum of the chloroform extract of chymotrypsin fragments

The spectrum shows the characteristic methylene and methine protons of OHB. The methyl protons are hidden under the resonances of impurities. Assignments: methylene protons form an octet at 2.45–2.65 ppm; methine protons form a multiplet at 5.23 ppm (56,57).

Figure 4. Representative single-channel current traces showing the effect of disulfide reducing agents on the narrow to large pore transition of M-OmpA

Each preparation was reconstituted in C₈E₄ micelles, incubated at 40 °C o/n to induce the narrow to large pore transition, and then cooled to room temperature and inserted into bilayers of DPhPC between aqueous solutions of 20 mM Hepes, pH 7.4, 1 M KCl at 22 °C. Top trace: 1 mM 2-ME was added *before* incubation at 40 °C; Middle trace: 1 mM DTT was added *before* incubation at 40 °C o/n. Bottom trace: 1 mM DTT was added *after* incubation at 40 °C o/n. The closed state is indicated by the bar at the right of each trace. The corresponding histograms from one minute of continuous recording show the distribution of conductance magnitudes.

Figure 5. Molecular model of the longest extracellular loop formed by residues 288–307 of the C-terminal domain of OmpA

The model is according to Stathopoulos (41). Red: positive residues; Blue: negative residues; Yellow: cysteine residues. The backbone is traced in green. Salt bridges are shown in grey ovals.

Figure 6. Representative single-channel current traces of M-OmpA showing the effect of urea on pore structure

Top trace: M-OmpA isolated using urea and 2-ME; Bottom trace: M-OmpA isolated using urea without 2-ME. Bilayers were formed from DPhPC between aqueous solutions of 20 mM Hepes, pH 7.4, 1 M KCl at 22 °C. The clamping potential was +100 mV with respect to ground (*trans*). The corresponding histograms from one minute of continuous recording show the distribution of conductance magnitudes. The bar at the right of each trace indicates the closed state.