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OmpA can form folded and unfolded oligomers

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ABSTRACT

The monomeric outer membrane protein OmpA from *Escherichia coli* has long served as a model protein for studying the folding and membrane insertion of β-barrel membrane proteins. Here we report that when OmpA is refolded in limiting amounts of surfactant (close to the cmc), it has a high propensity to form folded and unfolded oligomers. The oligomers exist both in a folded and (partially) unfolded form which both dissociate under denaturing conditions. Oligomerization does not require the involvement of the periplasmic domain and is not strongly affected by ionic strength. The folded dimers can be isolated and show native-like secondary structure; they are resistant to proteolytic attack and do not dissociate in high surfactant concentrations, indicating high kinetic stability once formed. Remarkably, OmpA also forms significant amounts of higher order structures when refolding in the presence of lipid vesicles. We suggest that oligomerization occurs by domain swapping favored by the high local concentration of OmpA molecules congregating on the same micelle or vesicle. In this model, the unfolded oligomer is stabilized by a small number of intermolecular β-strand contacts and subsequently folds to a more stable state where these intermolecular contacts are consolidated in a native-like fashion by contacts between complementary β-strands from different molecules. Our model is supported by the ability of complementary fragments to associate with each other *in vitro*. Oligomerization is probably avoided in the cell by the presence of cellular chaperones which maintain the protein in a monomeric state.

1. Introduction

Outer membrane protein A (OmpA) from *Escherichia coli* consists of a 171-residue N-terminal 8-stranded β-barrel transmembrane domain embedded in the outer membrane and a 154-residue C-terminal periplasmic domain [1]. OmpA is one of the most abundant structural proteins in the outer membrane of *E. coli* [2]. It is required for the stabilization of mating aggregates in conjugation [3,4], serves as a receptor for a number of bacteriophages [5–8] and acts as a low-permeability porin that allows slow penetration of small solutes [9]. Its biological importance combined with ease of expression, purification, reversibility of unfolding and high stability has made OmpA an excellent model to study the folding, membrane insertion and assembly of integral membrane proteins [10–17]. OmpA is typically purified in the urea-denatured state from inclusion bodies in *E. coli*, and can fold into its native structure by diluting out the urea in the presence of lipids or surfactants. Folding can be followed by *e.g.* far-UV circular dichroism, Trp fluorescence or an SDS-PAGE gel-shift assay, since the native state resists denaturation in SDS at room temperature and migrates faster than the denatured state in the full length form [18] (the situation is reversed for the transmembrane domain alone). Refolding in surfactants requires these surfactants to be present as micelles, *i.e.* at concentrations above the critical micelle concentration or cmc [15]. Micelle sizes vary depending on chain length and head group structure, and if micelles are smaller than the membrane-inserted β-barrel portion of the protein, it has been proposed that two or more micelles will have to coalesce to form larger structures in the mixed protein/surfactant micelles [15]. Membrane-incorporated and completely refolded OmpA is partially protected from trypsin digestion by the membrane in the same way as native OmpA in the outer membrane of *E. coli* [19,20]. This indicates that only the transmembrane domain folds in micelles, leaving the periplasmic domain folded outside the micelles.

OmpA belongs to the outer membrane porin protein family together with proteins such as OmpF and OmpC porins. While OmpA is believed to be monomeric *in vivo* and behaves like a monomeric protein *in vitro*, porins exist as trimers even in the presence of SDS [21]. There is also evidence that OmpA can forms homodimers, according to a blue native-PAGE study of protein complexes from the *E. coli* cell envelope [22]. Protein oligomers are essential for life, furthermore, the folding of membrane and oligomeric proteins has

Abbreviations: cmc, critical micelle concentration; FL-OmpA, full-length OmpA; OmpA, outer membrane protein A; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (sodium salt); SDS-PAGE, sodium dodcyl sulfate polyacrylamide electrophoresis; TM-OmpA, transmembrane OmpA

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important implications for both biotechnology and medicine [23]. Oligomerization of OmpA could conceivably stabilize the anchoring of the periplasmatic polyglycan matrix to the outer membrane [24–27]. Simulations of simple β-barrel membrane protein model systems indicate that oligomerization could be a general feature in the membrane [28]. Whether OmpA has genuine pore-forming activity is controversial. The high resolution X-ray structure of OmpA [26,29] reveals a monomeric structure without a free pathway between the external and periplasmic ends of the barrel, though there are several water-filled cavities inside the barrel. This does not indicate that OmpA could function as a pore. The low level of channel activity reported for OmpA [9,30] might therefore arise from a fraction of the molecules assuming a non-native conformation. In fact, single channel conductance measurements have revealed a permeability to ions which have led to the proposal that refolded OmpA forms ion channels with two conductance states in planar lipid bilayers [24]. In this model, the two-domain structure is a partially folded intermediate and forms a low conductance narrow pore that is kinetically stable at lower temperatures. Mature fully folded OmpA, which dominates at temperatures above 37 °C, is suggested to be a large high-conductance pore incorporating both β-barrel and periplasmic domain [31]. Consistent with this, the large pore was not formed when only the N-terminal domain was present [32]. A large pore structure for OmpA is consistent with structures of outer membrane porin protein family [33–36] and with known functions of OmpA as a receptor for bacteriophages [5–8], which suggest that it may serve as a conduit for large organic molecules such as single strand DNA. However, two-domain narrow-pore and one-domain large-pore conformers cannot be distinguished by their electrophoretic mobilities; both migrate as "native" proteins with similar mobility [32]. It has also been proposed that the large pores can oligomerize [37].

Here we report that OmpA forms dimers, trimers and higher-order oligomers when refolded from the urea-denatured state at concentrations around the cmc of the surfactant dodecyl maltoside (DDM). These oligomers are detected by SDS-PAGE as bands consistent with both native and (partially) denatured OmpA, and their interconversion is compatible with folding at different levels. We suggest that this phenomenon arises because the low surfactant micelle concentrations allow several OmpA molecules to interact within the same micelle and form higher order structures during the folding process. This does not happen at higher micelle:protein ratios where there is on average less than one OmpA molecule per micelle. We show that the native and denatured oligomers are easily destabilized by temperature or denaturants, indicating rather weak interactions between monomers within each oligomers. We speculate that oligomerization at different levels of folding occurs by different degrees of domain swapping, aided by the high propensity of β-strands to engage promiscuously in intermolecular contacts. The fact that this phenomenon also occurs in lipid vesicles indicates that it is a potential complication which Nature must safeguard against *in vivo*.

2. Materials and methods

2.1. Materials

Urea, Tris, Glycine, NaCl and EDTA were from Sigma (St. Louis, MO). Dodecyl maltoside (DDM) was from Anatrace (Maumee, OH). POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) and POPG (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (sodium salt) were from Avanti Polar Lipid (Alabaster, AL). Triton X-100 was from Fluka. All SDS-PAGE experiments were carried out using a 15% polyacrylamide separation gel prepared according to Laemmli [38]. Full-length OmpA, FL-OmpA (containing both periplasmic and membrane-bound domains) was produced as described [39]. The transmembrane domain of the OmpA gene, TM-OmpA (a construct containing residues 1–176 of the N-terminal domain without the preceding leader sequence, followed by the C-terminal sequence Arg-Ser-$(His)_6$) was cloned, expressed and purified as described [40].

2.2. Folding of FL-OmpA and TM-OmpA

160 μM FL-OmpA or TM-OmpA in 8 M urea was diluted out to a final concentration of 4 μM in different (0.05–7 mM) concentrations of DDM, 10 mM glycine, 2 mM EDTA, pH 10 and a residual urea concentration of 200 mM urea at 20 °C unless otherwise stated. After incubating for 2 days, 16 μl was taken out, mixed with 4 μl 5× sample buffer and subjected to SDS-PAGE without reducing agents and prior boiling unless specified. Different states were distinguished by the fact that folded FL-OmpA migrates faster than its unfolded counterpart; this migration order is reversed for TM-OmpA. In addition, refolding experiments were conducted in 0.3 mM DDM and buffer for 48 h either at 4–65 °C, 0–1 M NaCl (20 °C) or 0–1.5 M urea (20 °C). Band intensity was quantitated by scanning densitometry using a conventional scanner in combination with the programme ImageJ. Our calibration tests reveal a strict proportionality between loaded protein and band intensity over a 64-fold difference in concentration (based on 2-fold dilution steps); this calibration range covers the full range of intensities that we have recorded in our experiments. In addition to bands corresponding to unfolded and folded monomers and dimers of OmpA, higher oligomers were also produced to a small extent. For simplicity, we restricted the densitometry analysis to the bands corresponding to the monomer and dimer unless otherwise stated. In order to analyze the possible role of disulfide bond formation in oligomerization, refolding with or without 20 mM the reducing agent DTT was also performed in 0.4 mM DDM for 16 h and subsequently analyzed by SDS-PAGE with or without prior boiling for 5 min. 4 μM FL-OmpA was also folded in different concentrations of sonicated phospholipid vesicles composed of 92.5% POPC and 7.5% POPG (w/w) for 21 h (a reduction from the normal 2 days of incubation to reduce complications from possible oxidation of the unsaturated bond in the oleoyl chain), and subsequently analyzed by SDS-PAGE without prior boiling.

2.3. Folding kinetics of FL-OmpA

The folding kinetics of FL-OmpA in surfactant micelles was monitored *via* SDS-PAGE. Typically, FL-OmpA was diluted directly from 8 M urea to the refolding buffer containing 10 mM glycine, 2 mM EDTA, pH 10 and 0.3 mM DDM, giving a residual urea concentration of 0.2 M. At different time points, 16 μl of reaction mixture was immediately mixed with 4 μl 5× sample buffer (without DTT) to arrest folding and then analyzed by SDS-PAGE without prior boiling. The amount of folded and unfolded protein was quantified by scanning as above. Band intensities were fitted to a single exponential decay using the programme Kaleidagraph 4.0 (Synergy Software).

2.4. Pyrene fluorescence

Pyrene is a highly hydrophobic molecule with low (~1 μM) solubility in water. The ratio of the emission peaks at 372.5 (I_1) and 383.5 nm (I_3) can be used to evaluate the polarity of its environment and thus determine the cmc [41]. Different amounts of DDM were added to buffer (10 mM Glycine pH 10 and 2 mM EDTA). After equilibration for 30 min, pyrene was added from a 50 μM stock in ethanol to a final concentration of 1 μM. Fluorescence scans were performed from 360 to 410 nm using an excitation wavelength of 335 nm and excitation/emission slits of 5/3.5 nm.

2.5. Studies on the TM-OmpA dimer

TM-OmpA was folded from 8 M urea into refolding buffer to a final concentration of 125 μM TM-OmpA and 25 mM DDM, giving a protein:DDM molar ratio of 1:200. After incubation for 24 h at room