FOR THE RECORD

Effect of crowding by Ficolls on OmpA and OmpT refolding and membrane insertion

Cui Ye, Qian Chai, Meng Zhong, and Yinan Wei*

Department of Chemistry, University of Kentucky, Lexington, KY 40506

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Abstract: Folding of outer membrane proteins (OMPs) has been studied extensively *in vitro*. However, most of these studies have been conducted in dilute buffer solution, which is different from the crowded environment in the cell periplasm, where the folding and membrane insertion of OMPs actually occur. Using OmpA and OmpT as model proteins and Ficoll 70 as the crowding agent, here we investigated the effect of the macromolecular crowding condition on OMP membrane insertion. We found that the presence of Ficoll 70 significantly slowed down the rate of membrane insertion of OmpA while had little effect on those of OmpT. To investigate if the soluble domain of OmpA slowed down membrane insertion in the presence of the crowding agent, we created a truncated OmpA construct that contains only the transmembrane domain (OmpA171). In the absence of crowding agent, OmpA171 refolded at a similar rate as OmpA, although with decreased efficiency. However, under the crowding condition, OmpA171 refolded significantly faster than OmpA. Our results suggest that the periplasmic domain slows down the rate, while improves the efficiency, of OmpA folding and membrane insertion under the crowding condition. Such an effect was not obvious when refolding was studied in buffer solution in the absence of crowding.

Keywords: macromolecular crowding; outer membrane protein; protein folding; membrane insertion

Introduction

Gram-negative bacteria such as *Escherichia coli* contain two layers of membranes, separated by the periplasmic space. Proteins embedded in these two membranes are different. While the inner membrane contains mainly α -helical transmembrane proteins, the outer membrane proteins (OMPs) form β -barrels.¹

OMPs serve various functions that are crucial to cell viability and activity, including structural support, catalysis, and are involved in both active transport and passive diffusion. The biogenesis of OMPs is a complicated process.^{2–5} All proteins are synthesized by ribosomes in the cytosol. OMP preproteins containing N-terminal signal peptides are first translocated across the inner membrane through translocons. Once reaching the periplasm, the leader signal peptide of OMP is removed by signal peptidase. Molecular chaperones including Skp, DegP, and SurA stabilize OMPs in the periplasm and assist their folding and insertion into the outer membrane through the β -barrel assembly machinery.⁶

Most current understanding about OMP folding has been obtained from *in vitro* studies conducted in

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*Correspondence to: Yinan Wei, 305 Chemistry-Physics Building, University of Kentucky, Lexington, KY 40506-0055. E-mail: yinan.wei@uky.edu

Figure 1. OMP refolding and membrane insertion. A. Crystal Structures of OmpA (1QJP.pdb²⁵) and OmpT (1I78.pdb²⁶) viewed from the side. The oval in OmpA represents its periplasmic domain, the structure of which remains unknown. B. Representative SDS-PAGE images of OmpA and OmpT refolding experiment in the absence of Ficoll 70. BS: boiled sample. M: protein molecular weight marker. For OmpA, lanes 1 to 9 correspond to 0, 10, 20, 40, 80, 160, 320, 640, and 1410 min of incubation. For OmpT, lanes 1 to 11 correspond to 0, 30, 60, 120, 180, 240, 300, 360, 540, 900, and 1440 s of incubation. C. Kinetics of OmpA and OmpT refolding, in buffer (triangles), 5% Ficoll 70 (diamonds), 10% Ficoll 70 (circles), and 20% Ficoll 70 (squares). Data were averages of three independent experiments and fitted to a single exponential equation.

dilute buffer solution.^{7–13} However, the actual bioproduction of OMPs occurs in a very crowded environment. The intracellular space is filled with a variety of macromolecules including proteins, polysaccharides, nucleic acids, and lipids.^{14,15} The periplasm has been described as a “gel-like” environment with macromolecules occupying ~ 30% of the overall space.¹⁶ Under this crowded condition, proteins may behave differently. This phenomenon is called the “macromolecular crowding” effect, first proposed by Minton and his coworker in 1981.¹⁷ It is later summarized by Minton as “the influence of volume exclusion upon the energetic and transport properties of macromolecules within a crowded or highly volume-occupied medium.”¹⁸ The impact of macromolecular crowding on biological systems is attracting increased attention from the research community in recent years. Studies on soluble proteins have shown that the crowding condition affects the stability of proteins and alter their unfolding/refolding processes.^{19–24} In this study, we investigated the macromolecular crowding effect on the refolding and membrane insertion of OMPs, using OmpA and OmpT as model proteins [Fig. 1(A)^{25,26}] and Ficoll 70 as crowding agent to mimic the crowded condition in the periplasm. Ficoll 70 is a sucrose polymer that has been used as an inert cosolute in many studies investigating the crowding effect on protein properties.^{27–29}

Results and Discussion

Effect of the crowding condition on the rate of membrane insertion of OmpA and OmpT

To investigate the crowding effect on the folding and membrane insertion of OMPs, we monitored refold-

ing and membrane insertion of OmpA and OmpT in small unilamellar vesicles (SUV) solution in the absence or presence of Ficoll 70. Three different Ficoll 70 concentrations, 5, 10, and 20%, were used in this experiment. At concentrations above 20%, the high viscosity of the solution drastically decreased the efficiency of sonication and thus the formation of liposomes. To examine the effect of crowding on liposome formation, sizes of SUV vesicles prepared in the absence or presence of Ficoll 70 were measured using dynamic light scattering. Sizes of SUVs were in the range of 10–100 nm, and Ficoll 70 at concentrations up to 20% had little effect on the size distribution of SUVs (data not shown). Folding and membrane insertion of OMPs were observed using SDS-PAGE. Previous studies have shown that folded and unfolded OMPs usually migrate differently on SDS polyacrylamide gel.^{7–13} As shown in Figure 1(B), after Coomassie blue stain, two bands were clearly visible on the gel. The bands with lower and higher apparent molecular weight were the folded and unfolded species, respectively.

Intensities of bands were quantified using software ImageJ, and converted into fraction of folded protein.³⁰ Several studies have shown that folding kinetics of OMPs can be fitted using a single exponential equation.^{8,10,31} Therefore, we fitted the percent folding data over time using a single exponential equation [Fig. 1(C)]:

$$y = y_0 + Ae^{-kt}$$

where y is the folded fraction at certain time point, t is the time, and y_0 is the fraction folded as time approaches infinity. The rate constant k could be obtained through fitting. The rate constants of

Table I. Refolding Rate Constants (min^{-1})

OMP	No Ficoll 70	5% Ficoll 70	10% Ficoll 70	20% Ficoll 70
OmpA	0.016 ± 0.004	0.019 ± 0.005	0.0049 ± 0.0001	0.0020 ± 0.0002
OmpT	0.20 ± 0.04	0.14 ± 0.03	0.24 ± 0.03	0.18 ± 0.02
OmpA171	0.014 ± 0.002	—	—	0.028 ± 0.002

refolding obtained in the presence or absence of Ficoll 70 were shown in Table I. We found that low Ficoll 70 concentration (5%) had little effect on the refolding rate of OmpA. The rate constant decreased significantly in 10 and 20% Ficoll 70. However, Ficoll 70 at a concentration as high as 20% had little effect on the refolding rate of OmpT, whereas the overall folding efficiency decreased with the increase of Ficoll 70 concentration.

To evaluate if the effect of Ficoll 70 on the folding rate of OmpA was due to specific polar interaction, we examined OmpA folding in the presence of 20% sucrose (Fig. 2). Sucrose is the monomer of Ficoll 70. Because of its chemical similarity to Ficoll 70, sucrose has been used in several studies as a small molecule control to assess the contribution from specific interactions.^{32–35} The folding rate of OmpA in 20% sucrose ($0.016 \pm 0.002 \text{ min}^{-1}$) was the same as the folding rate of OmpA in buffer ($0.016 \pm 0.004 \text{ min}^{-1}$). This result suggests that the decrease of folding rate in the presence of Ficoll 70 is likely due to the volume exclusion effect.

Circular dichroism (CD) spectroscopy was used to characterize the structure of OmpA folded in the

presence or absence of 20% Ficoll 70 (Fig. 3). In addition, we have collected the CD spectrum of completely unfolded OmpA dissolved in 8 M urea. Absorption from urea restricted the useful wavelength window to above 205 nm. The spectra of folded OmpA were drastically different from the spectrum of unfolded protein, indicating the formation of secondary structures during folding. The negative peak between 210 and 220 nm indicated an abundance of β -sheet structure in folded OmpA. As revealed in Figure 1(C), OmpA refolding did not reach completion, potentially due to irreversible aggregation of folding intermediates during *in vitro* refolding. The samples used for the CD measurement were incubated under the refolding condition overnight. Approximately 92 and 83% of OmpA folded and inserted into membrane vesicles in the absence or presence of 20% Ficoll 70, respectively. This difference might explain the observed difference between the spectra of OmpA folded in the presence or absence of Ficoll.

Effect of the crowding condition on the rate of membrane insertion of the transmembrane domain of OmpA

OmpA contains a large C-terminal periplasmic domain.²⁵ To investigate the role of the periplasmic domain in the observed crowding effect, we constructed a plasmid encoding the sequence of just the transmembrane domain of OmpA (OmpA171). The transmembrane domain of OmpA has been shown to fold into an eight-stranded β -barrel, the structure of which has been determined by both X-ray

Figure 2. OmpA refolding and membrane insertion in 20% sucrose. A. A representative SDS-PAGE image. BS: boiled sample. Lanes 1 to 9 correspond to 0, 10, 20, 40, 80, 160, 320, 640, and 1410 min of incubation. B. Kinetics of OmpA refolding in 20% sucrose. Data were averages of three independent experiments and fitted to a single exponential equation.

Figure 3. CD spectra of unfolded OmpA dissolved in 8 M urea (dash line), and OmpA folded in the presence (grey) or absence (black) of 20% Ficoll 70.

Figure 4. Representative SDS-PAGE images of OmpA171 refolding and membrane insertion in the absence (A) or presence (B) of 20% Ficoll 70. BS, boiled sample. M, protein molecular weight marker. Numbers represent different incubation time (min). C. Kinetics of OmpA171 refolding in the absence (open squares) or presence (filled squares) of 20% Ficoll 70. Data were averages of three independent experiments and fitted to a single exponential equation. D. Representative SDS-PAGE images of OmpA171 and OmpA refolding in the absence of Ficoll. Higher molecular weight bands are clearly visible in OmpA171, but not in full length OmpA. Positions of folded (F) and unfolded (UF) proteins were marked.

crystallography and NMR.^{36–38} When reconstituted into proteoliposomes, the transmembrane fragment of OmpA forms ion conducting channels.³⁹ We purified unfolded OmpA171 and conducted refolding experiment under similar condition as the full-length OmpA. Folded OmpA171 and unfolded species were separated on SDS polyacrylamide gel, in which unfolded species moved faster [Fig. 4(A,B)].⁴⁰ In the absence of Ficoll 70, the folding and membrane insertion rate of OmpA171 was $0.014 \pm 0.002 \text{ min}^{-1}$, while the rate increased approximately twice on the addition of 20% Ficoll 70 ($0.028 \pm 0.002 \text{ min}^{-1}$), indicating that the addition of Ficoll 70 accelerated the folding and insertion process of OmpA171 (Table I). We also noticed that OmpA171 tended to aggregate. High molecular weight bands corresponding to different aggregation states emerged during extended incubation [Fig. 4(D)]. The presence of Ficoll 70 slightly increased the percentage of OmpA171 refolded correctly [Fig. 4(C)].

In summary, we used Ficoll 70 as a model crowding agent and studied the crowding effect on the folding and membrane insertion of OmpA and OmpT. First, we found that the folding and membrane insertion rate of OmpA decreased by eight-folds in the presence of 20% Ficoll 70, whereas the rate of OmpT folding was barely affected. The presence of 20% sucrose, the monomer of Ficoll 70, has no effect on OmpA refolding. In addition, we noticed that OmpT folded significantly faster than OmpA under the current experimental condition (Table I).

To investigate the role played by the OmpA periplasmic domain in the observed crowding effect, we created a truncated OmpA construct containing only the transmembrane domain and investigated its rate of folding and the corresponding effect of crowding. Interestingly, while OmpA171 and full-length OmpA had similar folding rate in dilute buffer solution, in the presence of 20% Ficoll 70, full-length OmpA refolded much slower than OmpA171. The periplasmic domain in full-length OmpA slowed down its folding and membrane insertion under crowding condition. In addition, we found that unfolded OmpA171 was less stable during the refolding process, aggregating and forming oligomeric species of high molecular weight over time. The periplasmic domain of OmpA apparently played a role in stabilizing the structure of OmpA folding intermediates, before their complete folding and insertion into the outer membrane. Our results are consistent with the conclusion of an earlier study, in which Fleming and Danoff⁴¹ suggested that the periplasmic domain of OmpA serves as a “chaperone” during its folding to keep the protein stable and soluble in the periplasm.

Materials and Methods

Protein cloning, expression, and purification

Genes encoding OmpA, OmpT, and OmpA171 without the signal peptide were amplified from the genomic DNA of *E. coli* K-12 and inserted into

plasmid pET22b, which introduced a six-histidine tag at the C-terminal of protein. Primers used in the construction of plasmids are: 5'-GCT CAT ATG GCT CCG AAA GAT AAC ACC TGG TAC-3' (OmpA forward), 5'-GCC TCG AGA GCC TGC GGC TGA GTT ACA ACG TC-3' (OmpA reverse), 5'-GAC ATA TGT CTA CCG AGA CTT TAT CGT TTA CTC-3' (OmpT forward), 5'-GAC TCG AGA AAT GTG TAC TTA AGA CCA GCA GTA G-3' (OmpT reverse), 5'-GAC ATA TGG CTC CGA AAG ATA ACA CCT GGT AC-3' (OmpA171 forward), and 5'-AAC TCG AGA CCG AAA CGG TAG GAA ACA CC-3' (OmpA171 reverse). Restriction enzyme digestion sites are underlined. Plasmid containing OMP gene was then transformed into *E. coli* strain ER2566 for protein expression. Cells were cultured at 37°C in Lysogeny Broth media containing 100 µg mL⁻¹ ampicillin to an OD_{600nm} of approximately 0.6. The expression of protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. After 3 h, cells were harvested by centrifugation at 7000g for 10 min at 4°C. Cell pellets were stored at -20°C or used immediately for purification.

The purification procedures for OmpA and OmpT were the same. Briefly, cell pellets were resuspended in a lysis buffer (30 mM Tris base, 0.5M NaCl, 1% Triton, 1 mM phenylmethylsulfonyl fluoride, pH 8.0) and then sonicated on ice for 15 min with 10-s on/off intervals to lyse cells. After centrifugation at 10,000g for 30 min at 4°C, the supernatant containing soluble proteins was discarded, whereas the pellet was dissolved in the sonication buffer (30 mM Tris base, 0.5M NaCl, 6M guanidine, pH 8.0) and sonicated again with the same setting. After sonication, the mixture was centrifuged at 25,000g for 20 min at 4°C. The supernatant containing solubilized OMPs was loaded to a Ni-nitrilotriacetic acid column, washed using a buffer containing imidazole (30 mM Tris base, 0.5M NaCl, 8M urea, 20mM imidazole, pH 8.0), and eluted using an elution buffer (20mM sodium acetate, 0.5M NaCl, 8M urea, pH 4.0). The pH of protein solution was then adjusted to 8.0 immediately after elution. OmpA171 was purified as described with slight modifications.¹⁰ Briefly, cell pellets were resuspended in the lysis buffer, sonicated on ice, and then centrifuged to obtain the inclusion body similarly as described for the purification of OmpA and OmpT. The inclusion body was washed twice using a wash buffer (20 mM sodium acetate, 0.5M NaCl, 2M urea, 1% Triton, pH 8.0) followed by centrifugation (2900g, 30 min, 4°C). Before refolding experiment, OmpA171 pellet was dissolved in a buffer containing 8M urea (2 mM EDTA, and 10 mM borate, pH 10.0). Purified protein was analyzed using SDS-PAGE and visualized after stained using Coomassie blue. Concentrations of all three proteins were determined using Pierce BCA Protein Assay Kit (Thermal Scientific, TX).

Preparation of small unilamellar vesicles

SUV was prepared as described with slight modifications.¹⁰ *E. coli* Lipid Total Extract (Avanti Polar Lipids) dissolved in chloroform was dried to form a thin film in a glass vial under a gentle stream of nitrogen. The lipid film was then placed in a vacuum oven at room temperature overnight to remove residual solvent. Before the insertion experiment, dry lipid film was hydrated in a borate buffer (10 mM Na-borate, 2 mM EDTA, pH 10) at a lipid concentration of 3.2 mg mL⁻¹ at 4°C overnight. Proper amount of Ficoll 70 (GE Healthcare, PA) were added to the borate buffer during hydration when indicated. SUV solution was prepared by sonicating hydrated lipid solution for 50 min using a Branson digital Sonic Dismembrator at 50% pulse cycle in an ice/water bath. The SUV solution was equilibrated overnight at 4°C and used within 48 h after preparation. The size of SUVs in solutions containing different amount of Ficoll 70 was determined using the Wyatt DynaPro dynamic light scattering system.

Folding and membrane insertion of OMPs

OMPs refolding experiments were performed following published protocol.¹⁰ Refolding was initiated by rapidly diluting a stock solution of OMP (in 8M urea) into a buffer solution containing 3.2 mg mL⁻¹ SUV (1M urea, 2 mM EDTA, 10 mM borate, pH 10.0, in the presence or absence of Ficoll 70) to a final concentration of 4 µM OMPs (25 µM for OmpA171) at 37°C. After folding was initiated, at different time point, small aliquots of the mixture were collected, quenched by adding SDS gel-loading buffer, and kept on ice. At the last time point, two aliquots were taken—one was treated similarly as other samples, and the other boiled for 5 min and used as a control. Samples were then analyzed using SDS-PAGE on a 12% gel. After electrophoresis, gels were stained with Coomassie blue. Gel images were analyzed using software ImageJ to obtain the percentage of refolding.³⁰ For OmpA and OmpT folding, fraction of folded protein was calculated using the following equation:

$$\text{Folding\%} = \frac{I_F}{I_T} \times 100\%$$

in which I_F and I_T are the intensities of bands corresponding to folded OMP at different time and the total OMP, respectively. In the case of OmpA171, folding% was calculated at the ratio of band intensities between the folded OmpA171 bands at each time point and total OmpA171 in the control lane.

CD spectroscopy

OmpA refolding was performed described above. After incubated at 37°C for 15 h, the extent of folding was examined using SDS-PAGE. Refolded samples were scanned from 205 to 250 nm using a JASCO

J-810 spectrometer. For the unfolded sample, OmpA dissolved in a buffer (30 mM Tris base, 0.5M NaCl, pH 8.0) containing 8M urea was used. Blank scans were collected under each buffer condition and subtracted from the corresponding plot.

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