Formation and function of bacterial outer membrane proteins- incorporated liposomes using an in vitro translation system

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Research Article

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Abstract

Outer membrane proteins (OMPs), located on the outer membrane of gram-negative bacteria, have a β -strand structure and form nanopores, which allow passage of ions, sugars, and small molecules. Recently, OMPs have been used as sensing elements to detect biological molecules. OMPs are normally expressed and purified from *E. coli*. Although the cell-free synthesis of OMPs, such as OmpA and OmpG, is achieved in the presence of liposomes and periplasmic chaperones, the amount of OmpA and OmpG incorporated into the nano-sized liposomes is not clear. In this study, after *in vitro* translation, the incorporation of OmpG into purified nano-sized liposomes, with various lipid compositions, was investigated. Liposomes containing the synthesized OmpG were purified using a stepwise sucrose density gradient. We report that liposomes prepared with the *E. coli* lipid extract (PE/PG) had the highest amount of OmpG incorporated compared to liposomes with other lipid compositions, as detected by recording the current across the OmpG containing liposomes using the patch clamp technique. This study reveals some of the requirements for the insertion and refolding of OMPs synthesized by the *in vitro* translation system into lipid membranes, which plays a role in the biological sensing of various molecules.

Introduction

Outer membrane proteins (OMPs) are inserted into the outer membrane of gram-negative bacteria^{1,2}. OMPs with 8–36 β strands can assemble into β -barrels that can form nanopores in the membrane, where the number of β -strands determine the diameter of the nanopores^{3,4}. Nanopores with varied diameters can allow passage of ions, sugars, and small molecules. Recently, OMPs are embedded into artificial cell membranes as sensing elements for detecting biological molecules using the patch-clamp technique, where biomolecules are detected based on the changes in the amplitude of current as they pass through the OMPs^{5,6}. Therefore, OMPs are important not only for biological functions but also for biotechnological applications. OMPs are normally expressed and purified from *E. coli*^{7,8,9,10} or are synthesized in an *in vitro* translation system^{11,12}. The *in vitro* translation system has some advantages, including rapid expression and purification, when compared to the *E. coli* expression system. Proteins with various characteristics, including those with different sizes or water-solubility, such as green fluorescent protein (GFP), connexin43, and human voltage-dependent anionic channel (hVDAC1), are synthesized using an *in vitro* translation system^{13,14,15,16}. Functional membrane proteins can be purified using the *in vitro* translation solution, that includes the nano-sized liposomes, as the membrane proteins are inserted directly into the liposomes during membrane protein synthesis¹⁴. The lipid membrane insertion of OmpA and OmpG using the *in vitro* translation system is investigated based on the composition of the liposomes and the presence of periplasmic chaperones such as Skp, DegP, and SurA^{11,12}. Although the synthesis of OmpA and OmpG using the *in vitro* translation system is carried out in the co-presence of the liposomes and the periplasmic chaperones, the exact amount of OmpA and OmpG that is incorporated in the nano-sized liposomes has not been clear because the liposome solution still contained the unincorporated proteins and the periplasmic chaperones, which were not purified after

the *in vitro* translation^{11,12}. The dynamics of the nanopore formation from OMPs synthesized by *in vitro* translation is also not clear.

In this study, the incorporation of OmpG into nano-sized liposomes, containing various lipid compositions, is investigated using purified nano-sized liposomes after *in vitro* translation (Figure 1). OmpG contains an antiparallel β -barrel structure consisting of 14 transmembrane strands¹⁷. To evaluate the amount of OmpG incorporated into the nano-sized liposomes produced using the *in vitro* translation solution, the liposomes are purified by a stepwise sucrose density gradient. The efficiency of incorporation of OmpG is confirmed by changing the lipid components and studying the diameter of the ensuing nano-sized liposomes. Finally, to investigate the nanopore formation of OmpG synthesized by *in vitro* translation, the ion currents of OmpG, which is incorporated into the bilayer lipid membrane (BLM) by fusing between the OmpG liposome and the BLM, are measured in the artificial cell membranes using the patch clamp system.

Results And Dissuasion

Expression of OmpG using in vitro translation system

OmpG, with or without addition of 20 mg/mL of a nano-sized liposome solution, was synthesized using a cell-free synthesis system. Fig. 1 shows the results of the SDS-PAGE analysis and western blot analysis of the OmpG synthesis solution. A band of OmpG was observed at approximately 30 kDa, which was not observed in the solution of the *in vitro* translation system without OmpG DNA (Figure 2 (a), Figure S1). The band of OmpG was detected by western blot analysis using an anti-His tag antibody because the His x 6 tag was conjugated at the N-terminal of OmpG (Figure 2 (b), Figure S2). Although the amount of OmpG synthesized in solution with the nano-sized liposomes was lower than that without the nano-sized liposomes, a sufficient amount of OmpG in the solution with nano-sized liposomes was also synthesized.

Incorporation of OmpG synthesized by the in vitro translation system into nano-sized liposomes

To investigate whether OmpG synthesized using the *in vitro* translation system was directly incorporated into the nano-sized liposome membrane, OmpG was synthesized into a cell-free synthesis solution containing various liposome compositions (DOPC, DLPC, DOPE/DOPG (7:3wt/wt%), or *E. coli* polar lipid extract (PE/PG/CA (67:23.2:9.8 wt/wt%))). After synthesis for 4 h at 37 °C, the synthesis of OmpG containing various liposome compositions was confirmed by SDS-PAGE. The bands of OmpG were detected in all samples containing nano-sized liposomes (Figure 3 (a), Figure S3 (a)). The bands of the samples were mixed in the OmpG-incorporated liposomes, the OmpG into the solution, and the components of cell-free synthesis. Therefore, to confirm the incorporation of OmpG into liposomes, liposomes containing OmpG were separated from the synthesized samples using a sucrose density gradient centrifugation method. The lipid concentrations in the purified liposome samples were

determined by measuring the concentrations of PC and PE. The bands of OmpG from the purified liposome compositions were detected by SDS-PAGE analysis when the liposome solution at a constant lipid concentration was applied to the SDS-PAGE gel. The OmpG band was only detected at approximately 30 kDa. These band positions of the OmpG, including the nano-sized liposomes, corresponded to the position of the band of the OmpG with the native conformation, suggesting that the OmpG, which is incorporated into nano-sized liposomes, is the folded OmpG¹⁸ (Figure 3 (b), Figure S3 (b)). The amount of OmpG incorporated into the nano-sized liposomes was as follows: large amounts of OmpG were incorporated into E. coli lipid liposomes, followed in order by DOPE/DOPG (7:3) liposomes, DOPC liposomes, and DLPC liposomes. Compared to DOPC liposomes and DOPE/DOPG (7:3) liposomes, the amount of OmpG incorporated into the DOPE/DOPG (7:3) liposomes was larger than that into the DOPC liposomes (Figure 3 (b), Figure S3 (b)). The tendency of incorporation of OmpA into the nano-sized liposomes was the same as the amount of OmpG incorporated into the liposomes (Figure S4). Although the length of the fatty acid chain is the same, only DOPG has a negative charge. Compared with E. coli lipid liposomes and DOPE/DOPG (7:3) liposomes, the amount of OmpG incorporated into the E. coli lipid liposome was larger than that incorporated into the DOPE/DOPG liposomes. The surface charge of liposomes, including E. coli. lipid and DOPE/DOPG are negative, and the length of the fatty acid chain is different between the E. coli lipid (containing various lengths of the fatty acid chain) and DOPE/DOPG (containing one length of the fatty acid chain). These results indicate that the incorporation of OmpG influences the surface charge and variability in the length of the fatty acid chain of the liposomes. In previous studies of OmpA and OmpG synthesized by the *in vitro* translation system, the SDS-PAGE analysis of the OmpA and OmpG-containing liposomes that was not purified after the *in vitro* translation was difficult for estimating the amount of OmpA and OmpG incorporated into the nano-sized liposome^{s11,12}. In this study, with the use of purified liposomes, the amount of OmpG incorporated in nano-sized liposomes, with various lipid components, was estimated for the first time. Although distinguishing between the OMPs embedded in the liposomes and those present free in the solution is difficult using previous methods, this experiment shows that the amount of OmpG inserted into the E. coli lipid liposomes was the largest, which were in the folded state without the presence of periplasmic chaperones such as Skp, DegP, and SurA.

Next, when the liposomes were added to the solution of the *in vitro* translation system before or after the synthesis of OmpG, the differences in the amount of OmpG incorporated into the E. coli lipid or DOPE/DOPG liposomes were investigated after the stepwise sucrose density gradient of the liposomes (Figure 3 (c), Figure S3 (b)). In case of the addition of the liposomes before the OmpG synthesis, the band of OmpG on the E.coli lipid or DOPE/DOPG liposomes was detected by SDS-PAGE analysis. In contrast, in the case of the addition of liposomes after OmpG synthesis, the band of OmpG on these liposomes was not detected. This result suggests that OmpG was incorporated into liposomes during OmpG synthesis. When membrane proteins were synthesized by the *in vitro* translation system, membrane proteins were incorporated into liposomes during their synthesis; in other words, the membrane proteins were aggregated in the absence of the liposomes during the cell-free synthesis¹⁴. Therefore, cell-free synthesis

of functional membrane proteins requires the coexistence of liposomes. These results corresponded with previous results on membrane protein synthesis using an *in vitro* translation system¹⁹.

Electrophysiological measurement of OmpG on the nano-sized liposomes

To confirm the formation of nanopores of OmpG into the nano-sized liposomes, the current signals of the OmpG were measured by a BLM chip that was connected to a patch clamp amplifier²⁰. The BLM was formed using the droplet contact method²¹. OmpG-containing liposomes were initially added to the droplets. By the fusion of liposomes with BLM, OmpG was incorporated into the BLM. We obtained an OmpG current amplitude of approximately 150 pA at +100 mV with 1 M KCl [Figures 4 (a) and (b)]. This amplitude was similar to that previously reported for OmpG channels in the BLM system²². This open-close signal shape is specific to OmpG. The specific current amplitude of OmpA in the nanosized liposomes was also detected (Figure S5). To the best of our knowledge, this is the first report of the ion current detection of Omp synthesized using an *in vitro* translation system. These results suggest that OmpG, which is incorporated into nano-sized liposomes, has a native conformation.

Conclusion

In this study, we show that OmpG incorporated into nano-sized liposomes can only be quantified by stepwise sucrose density gradient, which can remove the liposome-free OmpG and other molecules of the *in vitro* translation system from the OmpG-containing liposomes. Therefore, the amount of OmpG that is incorporated into the nano-sized liposomes when synthesized by the *in vitro* translation system changed with different lipid compositions, including the charge of the head group and the length of the acyl group. The liposomes produced with the *E. coli* lipid composition (PE/PG with various acyl group lengths) had the highest amount of OmpG incorporated compared to liposomes with other lipid compositions. The native conformation of OmpG incorporated into liposomes was revealed by patch-clamp analysis. This study reveals some of the requirements for the insertion and refolding of OMPs synthesized by the *in vitro* translation system into lipid membranes, which plays a role in the biological sensing of various molecules.

Materials And Methods

Reagents

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (DOPG), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), and *E. coli* extract polar were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). PURE^{flex}2.0 was purchased from Cosmo Bio (Tokyo, Japan).

Synthesis of OmpG using the *in vitro* translation system

OmpG was synthesized using the PURE[®]frex2.0 system. Solution I (6.25 μ L), solution II (0.625 μ L), solution III (1.25 μ L), OmpG and OmpA-coded DNA (1 μ L; 0.5 mg/mL), and dH₂O (3.375 μ L) were added to the PCR tube. The mixture was incubated for 4 h at 37 °C.

Synthesis and purification of OmpG containing the liposome solution using *in vitro* translation system

The lipids (DOPC, DLPC, DOPG/DOPE, and E. coli lipid) dissolved in chloroform were evaporated under flowing argon gas until lipid films formed at the bottom of the glass tube. The lipid films were hydrated by adding 20 mM HEPES/140 mM NaCl (pH 7.4) and then vortexed. Next, 20 mg/mL liposomes were prepared. To obtain monodisperse nano-sized liposomes, the liposome solution was filtered using an Avanti Mini-extruder with polycarbonate membranes of 100 nm diameter apertures²³. OmpG and OmpA were synthesized using PURE[®]frex2.0 in the presence of nanosized liposomes. A total of 12.5 μ L of solution I, 1.25 μ L of solution II, 2.5 μ L of solution III, 2 μ L of DNA, 6.75 μ L of dH₂O, and 10 μ L of 20 mg/mL nanosized liposomes were added to the PCR tube. The mixture was incubated for 4 h at 37 °C. The OmpG and OmpA-incorporated liposomes were separated using a stepwise sucrose density gradient [1.4 M, 0.8 M, and 0.5 M sucrose in 50 mM HEPES] at 40,000 rpm for 3 h at 4 °C (S52ST rotor, himac, Tokyo, Japan), and the OmpG- and OmpA-containing liposome fractions were collected.

The phosphocholine (PC) concentration for DOPC and DLPC liposomes, and the phosphoethanolamine (PE) concentration for DOPG/DOPE and E. coli lipid liposomes were measured. Each liposome that was adjusted for the lipid concentrations was analyzed by electrophoresis. One volume of sample buffer (Laemmli Sample Buffer, BIO-RAD, Hercules, CA, USA) was added to the OmpG and OmpA liposome samples. These mixtures were separated on a 15% SDS-PAGE gel, which was immersed in Oriole Fluorescent Gel stain (BIO-RAD). The bands of OmpG and OmpA were detected using a gel imaging system (LuminoGraph I, ATTO, Tokyo, Japan).

Electrophysiological measurement of OmpG

Eight microliters of 20 mg/mL DOPC solution dissolved in n-decane was added to each double well. Next, 23 μ L of buffer solution (10 mM HEPES/1 M KCl, pH 7.4) containing the OmpG liposomes was added to each well. The BLM spontaneously formed into an aperture diameter of 500 μ m on a separator integrated into the double well. OmpG was incorporated into the BLM by fusing the liposomes and BLM. This double-well device was connected to a portable patch-clamp amplifier (pico2). The current signals of OmpG were recorded using a patch clamp amplifier with a 1 kHz low-pass filter at a sampling frequency of 5 kHz (Tecella JET, California, USA). The measurement temperature was 20-23 °C. The current signals were analyzed using the pCLAMP software program (Molecular Devices, California, USA).

Declarations

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Author contributions

K.K. designed the research; K.K. performed the experiments and analyzed the data. K.K. wrote the paper.

Competing interests

The author declares no competing interests.

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Figures

Figure 1

Schematic representation of formation and function of OmpG liposomes using the *in vitro* translation system.

Figure 2

(a) SDS-PAGE analysis and (b) western blot analysis of OmpG synthesized by the *in vitro* translation system. Lane 1: protein molecular weight ladder, lane 2: Purefex solution (+ OmpG encoded DNA) with nano-sized liposomes, lane 3: Purefex solution (+ OmpG encoded DNA), lane 4: Purefex solution (- OmpG encoded DNA). Black triangle represents OmpG band.

Figure 3

SDS-PAGE analysis of before (a) and after (b) stepwise sucrose density gradient purification of OmpG synthesis containing nano-sized liposomes. Lane 1: protein molecular weight ladder, lane 2: Purefex solution (+ OmpG encoded DNA) with DOPC liposomes, lane 3: Purefex solution (+ OmpG encoded DNA) with DLPC liposomes, lane 4: Purefex solution (+ OmpG encoded DNA) with E. coli lipid liposomes, lane 5: Purefex solution (+ OmpG encoded DNA) with DOPE/DOPG liposomes, and (a) lane 6: Purefex solution (+ OmpG encoded DNA) or (b) lane 6: purified OmpG expressed by E. coli. (c) SDS-PAGE analysis of OmpG into nano-sized liposomes by addition of nano-sized liposomes before or after OmpG synthesis. Lane 1: protein molecular weight ladder, lane 2: Purefex solution (+ OmpG encoded DNA) by adding E.coli lipid liposomes before OmpG synthesis, lane 3: Purefex solution (+ OmpG encoded DNA) by adding DOPE/DOPG liposomes before OmpG synthesis, lane 4: Purefex solution (+ OmpG encoded DNA) by adding E. coli lipid liposomes after OmpG synthesis, lane 5: Purefex solution (+ OmpG encoded DNA) by adding DOPE/DOPG liposomes after OmpG synthesis, and lane 6: purified OmpG expressed by E. coli.

Black triangles represent OmpG band. Although there is separation between lane1-4 and lane5,6, the same gel of lane1-6 was conducted.

Figure 4

(a) Single channel current recording of OmpG incorporated into nano-sized liposomes (applied voltage: +100 mV). (b) Histogram of OmpG current amplitude.

Supplementary Files

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