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Quantitative analysis of protein orientation in membrane environments by kinase activity

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AgrC is an integral membrane receptor protein with histidine kinase activity in the accessory gene regulator (agr) quorum-sensing system of *Staphylococcus aureus*. In this study, proteoliposomes were used as a model to investigate AgrC orientation. Many approaches have been described to determine membrane protein orientation, but they are often complicated and time consuming. In this study, AgrC orientation in liposomes was determined by thiol-reactive reagent labeling and a kinase activity assay. Our results suggest use of a kinase activity assay could get an accurate percentage of functional protein orientation and only cost nearly one-sixth of the time compared with the method based on thiol-reactive reagent labeling. We present an effective and rapid method for determining the orientation of membrane protein kinases like AgrC.

[**Key words:** AgrC; Protein orientation; Liposome; Thiol-reactive reagent; Kinase activity]

Staphylococcus aureus is a human pathogen that causes many infections, including respiratory disease, skin infections, and food poisoning (1,2). Virulence factors produced by *S. aureus* are strongly related to the accessory gene regulator (agr) quorum-sensing system. AgrC is an integral membrane-embedded histidine kinase that has been described as an essential sensor for recognition and transduction of environmental signals to the cytoplasm (3–5). With the emergence of vancomycin resistant *S. aureus*, treatment of *S. aureus* infection has become a difficult clinical problem, and there is a distinct need to investigate signal transduction mechanisms responsible for pathogenicity and drug resistance to identify new drug targets and antimicrobial agents. AgrC is a promising novel antibacterial target that could combine with antimicrobial agents so as to prevent *S. aureus* infection (6,7).

Cell membranes consist of a phospholipid bilayer with embedded proteins. Studying proteins *in situ* is challenging due to the cell membrane's complex composition. Many researchers have attempted to reconstitute membrane proteins into artificial membranes like liposomes, which is a powerful tool in determining the structure and function of membrane proteins (8,9). The activities of many membrane proteins are only expressed when correctly inserted and oriented into a liposome (10). More than 50% of anticipated future drugs target membrane proteins, so knowing the orientations and target locations of membrane proteins is an extremely important goal (11). Several studies have addressed AgrC transmembrane topology and ligand specificity (12,13), but there are many unresolved questions about how the AgrC kinase activity and conformation are regulated by extracellular signals that could be resolved in inserting AgrC into a liposome.

Determination of membrane protein orientation by traditional methods used one-sided inhibitors and fluorescence probes to label the protein of interest, and the results were mostly qualitative. The objective of this study was to insert AgrC into a relevant proteoliposome model to determine AgrC's orientation in the membrane and investigate how different variables affected orientation. Topology modeling showed that AgrC consists of seven N-terminal transmembrane segments (residues 1–205) and a C-terminal cytoplasmic kinase domain (residues 206–430). There are two cysteine residues in the AgrC amino acid sequence: one in the transmembrane domain (residue 91), and the other is in the C-terminal cytoplasmic domain (residue 337) (12). In this work, AgrC orientation was determined by using fluorescence probe to label the cysteine residue located in C-terminal cytoplasmic domain and detecting kinase activity which associated with the kinase domain respectively. The objective of our study was to establish an efficient and rapid method to determine the orientation of functional AgrC in the membrane environment quantitatively.

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MATERIALS AND METHODS

Materials Fos-Choline-12 (Fos) was from Affymetrix. 1,2-Dipalmitoyl-sn-glycero-3-phosphate (DPPA), Dioleoyl-phosphatidyl-choline (DOPC) and cholesterol (chol) were from Avanti Polar Lipids. Thiol-reactive reagents 4-acetamido-4′-maleidylstibene-2, 2′-disulphonicacid (AmdiS), and 5-Iodoacetamidefluorescein (5-IAF) were from Invitrogen. Kinase-Glo Luminescent Kinase Assay Kit was from Promega. Bio-beads SM-2 were from Bio-Rad.

Overexpression and purification of AgrC AgrC expression and purification was as previously described (14). Briefly, *Escherichia coli* C43(DE3) expressing either AgrC or AgrC-GFP were lysed in PBS buffer containing Fos detergent at 10× CMC. Immobilized metal affinity chromatography (IMAC) and size-exclusion chromatography (SEC) were used to purify the proteins. Fractions were analyzed by SDS-PAGE which showed that protein purity was at least 95%. Purified protein was concentrated using an Amicon Ultrafree centrifugal filter (Millipore Corporation, Billerica, MA, USA) with 10 kDa cutoff. Protein concentration was determined by BCA assay (Sangon, Shanghai, China). AgrC protein concentration was 2.5 mg/mL and AgrC-GFP protein concentration was 2 mg/mL.

Liposome preparation A homogeneous mixture of different lipids is necessary for proper liposome formation. Different lipids [2 mM, DPPA: DOPC: chol in a 2: 2: 1 M ratio] were dissolved in chloroform and methanol (2:1 v/v). Once the lipid solution turned clear, the solvent was removed under a gentle nitrogen stream to yield a lipid film. The residual organic solvent was removed by placing the vial in a vacuum overnight. The resulting lipid films were hydrated at a temperature above the gel-liquid crystal transition temperature (Tc) of the lipids by adding a preheated 10 mM HEPES buffer (pH = 7.4) to the vial. The mixture was incubated at the same temperature for 1 h and vortexed at 10 min intervals to form multilamellar vesicles (MLVs). A bath sonicator was used to disrupt MLVs into small, unilamellar vesicles (SUV), by sonicating suspensions at 30 W for 15 min above the Tc of the lipids used. Liposomes were characterized by transmission electron microscopy (TEM, JEM-2100, JEOL, Japan) and dynamic light scattering (DLS, SZ-100, Horiba, Japan).

Reconstitution of proteins into liposomes To reconstitute AgrC and AgrC-GFP in liposomes, protein-free liposomes (2 mM) were solubilized with Fos detergent (50 mg/mL) until the onset of solubilization (condition A) or complete solubilization (condition B) was reached, respectively. After the mixtures equilibrated for 30 min at room temperature, purified proteins were added at a lipid to protein ratio of 250:1 and were incubated for 40 min at room temperature. Detergent was removed by adsorption on SM2 Bio-Beads (15). Briefly, 10 mg of Bio-Beads were added directly to the lipid−protein−detergent solution and agitated for 1 h at 4°C. An equal amount of beads was added for an additional 1 h incubation at 4°C. Samples were incubated with another 10 mg of beads overnight at 4°C. Samples were incubated with a fresh 10 mg bead aliquot for 3 h at 4°C to ensure complete detergent removal. After reconstitution, a drop of proteoliposomes was put on a glass slide, covered with a coverslip, and immediately imaged using a fluorescent microscope (B-51, Olympus, Japan) with magnification of 1000 × ($10\times_{\text{Eyepiece}} \times 100\times_{\text{Objective}}$) and blue excitation light (wavelength 488 nm).

Determining AgrC orientation by thiol-reactive reagent AgrC contains a unique cysteine at amino position of 337 in the cytoplasmic domain. AgrC orientation in liposomes was determined as previously described (16). Two kinds of thiol-reactive reagents (5-IAF and AmdiS) were used. After incubation with each thiol-reactive reagent, proteoliposomes were washed by dilution to 3 mL in HEPES buffer and then centrifuged for 1 h at 200,000 ×*g*, 4°C at least two times. Pellets were resuspended in HEPES buffer. After reactions, fluorescence was measured using a Synergy2 Multi-Mode Microplate Reader (BioTek, USA). Each experiment was repeated in triplicate three independent times. Results are presented with standard deviation (S.D.).

Determining AgrC orientation by kinase activity AgrC has a kinase active site in the cytoplasmic C-terminal domain (12). AgrC orientation in the liposome was evaluated by assaying for kinase activity. To determine if AgrC was oriented with the C-domain on the extracellular side, proteoliposomes were directly incubated with ATP to interact solely with the external protein (a_1) and an equal volume of empty liposomes was used as a control (a_0). To determine total protein kinase activity, 50 mg/mL Fos was added to solubilize the proteoliposome for 10 min followed by kinase activity detection (b_1) and equal volume of empty liposomes with the same treatment was used as a control (b_0). Kinase activity was detected using the Kinase-Glo Luminescent Kinase Assay Kit in 96-well white plates containing 5 μM ATP, 10 mM $MgCl_2$, and 150 pmol AgrC protein in 50 μL kinase reaction volumes in 10 mM HEPES buffer. Kinase reactions were incubated for 10 min at 37°C and followed by addition of 50 μL of ATP detection reagent for another 10 min at 37°C. Relative light units (RLU) were measured using the Synergy2 Multi-Mode Microplate Reader (BioTek). RLU was inversely related to kinase activity. Each experiment was conducted in triplicate 3 times, and results are presented with S.D. The AgrC with cytoplasmic-oriented C-domain (ω) was calculated using the following formula:

$$\omega = 1 - \frac{a_0 - a_1}{b_0 - b_1} \times 100\% \quad (1)$$

RESULTS AND DISCUSSION

Proteoliposome preparation To determine the AgrC's orientation, AgrC and AgrC-GFP proteins were purified and inserted into prepared liposomes.

We first prepared liposomes and characterized the morphology, particle size, and surface potential of liposomes by TEM and DLS. Liposomes had an average diameter of 132 nm (Fig. 1A and B). The zeta potential is related to the stability of colloidal dispersions and liposomes with a high zeta potential are electrically stable (17). As detected by DLS, our liposomes had a −61 mV zeta potential, suggesting our liposomes were very stable. Detergent-mediated reconstitution was used to prepare proteoliposomes containing AgrC (18). Fos detergent was used to disintegrate the structure of liposomes before protein insertion. To monitor the state of solubilization of our liposomes, turbidity of liposome suspensions was measured at 400 nm as a function of detergent concentration described by the three stage model (Fig. 2A) (19–22). In stage I, the detergent intercalates into the lipid bilayer until the liposomes are saturated, which is described as the onset of solubilization (condition A). During this stage, liposomes are not disrupted and there is little change in turbidity. During stage II liposomes are gradually solubilized into small lipid−detergent micelles, and turbidity decreases significantly. Stage III is characterized by the complete solubilization of all liposomes into lipid−detergent mixed micelles and the solution becomes optically transparent, referred to as complete solubilization (condition B) (22). Then, the AgrC or AgrC-GFP proteins were incorporated directly into liposomes at the onset

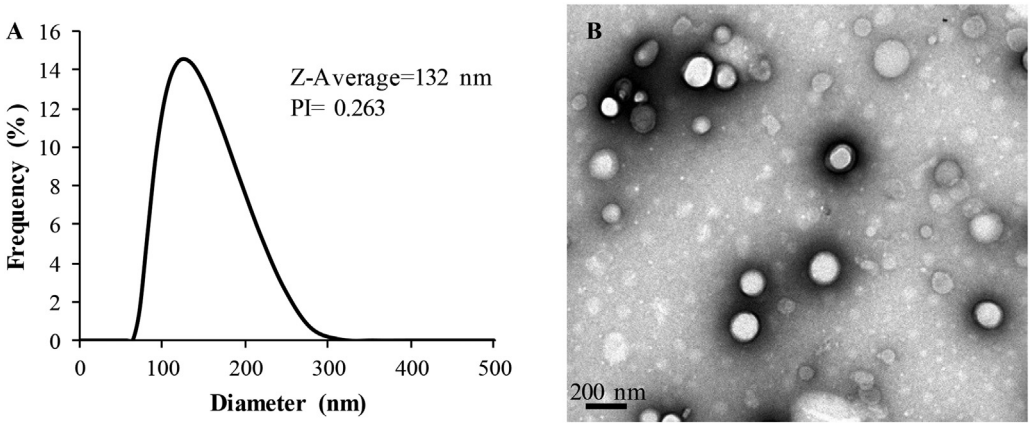

FIG. 1. Properties of prepared liposomes. (A) Size distribution of liposomes. (B) Electron micrograph of prepared liposomes.