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Structural basis for membrane association and catalysis by phosphatidylserine synthase in *Escherichia coli*

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Phosphatidylserine synthase (PssA) is essential in the biosynthesis of phosphatidylethanolamine, a major phospholipid of bacterial membranes. A peripheral membrane protein PssA can associate with the cellular membrane in its active state or exist in the cytosol in an inactive form. The membrane-bound enzyme acts on cytidine diphosphate diacylglycerol (CDP-DG) to form cytidine monophosphate and a covalent intermediate, which is subsequently targeted by serine to produce phosphatidylserine. Here, we present two crystal structures of *Escherichia coli* PssA, one complexed with CDP-DG and the other without. The lipid-bound structure mimics the Michaelis complex before the formation of a covalent intermediate, revealing key determinants for substrate recognition and catalysis. Notably, membrane-free PssA is in a monomer-dimer equilibrium, with only the monomer capable of associating with the membrane, suggesting a regulatory mechanism for phospholipid biosynthesis dependent on the oligomerization state of the enzyme.

INTRODUCTION

Phospholipids are amphipathic in nature, composed of hydrophobic acyl chains and hydrophilic glycerol and head groups that enable the formation of bilayers in biological membranes. These bilayers play a crucial role in signal transduction and cellular phenomena, such as maintaining the permeability barrier, providing structural integrity, and facilitating signal transduction (1-4). In bacteria, the cellular membrane is composed of three major phospholipids: phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL). Among these, PE is the most abundant species in *Escherichia coli*, comprising ~75 to 85% of total membrane lipids (5), which plays key roles in regulating protein synthesis and the activity of membrane-bound proteins (5, 6).

The biosynthetic pathway of PE in E. coli is well characterized (Fig. 1A). First, glyceraldehyde-3-phosphate undergoes two successive acylation steps, mediated by PlsB (7) and PlsC (8), leading to the formation of phosphatidic acid (PA). Subsequently, cytidine diphosphate diacylglycerol (CDP-DG) synthase (CdsA) assembles CDP-DG from PA and cytidine triphosphate (9). PE is then synthesized by phosphatidylserine synthase (PssA) and phosphatidylserine decarboxylase (PSD) (10), where PssA converts the CDP-DG and L-serine into phosphatidylserine (PS), while PSD subsequently decarboxylates PS to yield a zwitterionic phospholipid, PE (11, 12). Meanwhile, acidic phospholipids PG and CL are synthesized from CDP-DG by phosphatidylglycerophosphate synthase (PgsA) (13), phosphatidylglycerophosphate phosphatases (PgpABC) (14), and CL synthases (ClsABC) (15). Notably, PssA plays a key role in balancing the biosynthesis of zwitterionic and acidic phospholipids through cross-feedback regulation, which is activated by the acidic phospholipids in the cell membranes (16).

There are two subclasses of PssA: type I and type II. Type I PssA is primarily found in γ -proteobacteria and certain species of β proteobacteria, including various pathogenic bacteria such as *E. coli*, *Klebsiella michiganensis*, *Providencia stuartii*, *Salmonella typhimurium*, *Haemophilus influenzae*, *Vibrio cholerae*, and *Yersinia pestis* (fig. S1),

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which is essential for vitality in these organisms (17, 18). Type I PssA belongs to the phospholipase D (PLD) superfamily (19, 20), which includes PLD, ClsABC, envelope proteins from poxviruses, Yersinia murine toxin, and several endonucleases (19). A distinctive feature of this superfamily is the presence of one or two copies of the conserved HxKxxxxD (HKD) motif, with type I PssA containing two such conserved motifs (19, 21). Studies on the PLD from Streptomyces have led to the proposal of a general reaction mechanism for the PLD superfamily, characterized by the formation of a phosphohistidine intermediate, which subsequently undergoes hydrolysis to produce PA (22). A comparable reaction pathway is postulated for the type I PssA (23, 24), although structural evidence supporting this model is limited. A comparable reaction pathway is postulated for at the phosphorus center in PS (Fig. 1B). First, a histidine residue from the N-terminal HKD motif attacks the β-phosphate of CDP-DG, resulting in a release of cytidine monophosphate (CMP) and the formation of a phosphatidyl-histidyl covalent intermediate (21, 25). Subsequently, the second substrate Lserine attacks the phosphorus atom of the substrate-enzyme intermediate, leading to the production of PS with the concurrent release of the histidine residue (23). On the other hand, type II PssA is conserved in Gram-positive bacteria, lower eukaryotes, and archaea (fig. S1) (26-28). This subclass functions as a transmembrane protein within the CDP-alcohol phosphotransferase family, exhibiting a unique catalytic mechanism distinct from that of type I PssA. Type II PssA catalysis follows a sequential ordered Bi-Bi mechanism, which results in inversion at the phosphorus atom (27, 29). In addition, type II PssA requires metal for catalytic activity in contrast to Type I PssA (26, 27).

The kinetic characteristics of *EcPssA*, representative of the type I PssA enzymes from *E. coli*, have been the subject of thorough investigations. This enzyme is characterized by two HKD motifs, located at residues H138-K140-D145 and H357-K359-D364 (*19*). During catalysis, a conserved histidine residue serves as a nucleophile, initiating an attack on the phosphorus atom of a lipid substrate and leading to the formation of an enzyme-lipid intermediate. The enzymatic function is further facilitated by the interaction of a conserved aspartic (or sometimes glutamic) acid residue. A lysine residue is also implicated in forming a salt bridge with the phosphate group of the lipid substrate. Nonetheless, definitive identification of the pivotal residues within these HKD motifs remains elusive, primarily due to



Fig. 1. Biosynthetic pathway of glycerophospholipids in *E. coli* involving phosphatidylserine synthase (PssA). (A) PE biosynthetic pathway. Sequential acylations by PIsB and PIsC lead to the formation of PA. Subsequently, the CMP moiety is attached by CdsA to form CDP-DG. PssA then catalyzes the replacement of the head group with serine to generate PS, which is lastly decarboxylated by PSD to yield PE. (B) The catalytic mechanism of *Ec*PssA follows a ping-pong reaction, where an initial nucleophilic attack by a histidine residue on the substrate leads to the formation of a covalent phosphatidyl-enzyme intermediate. This intermediate is then subsequently substituted by serine, resulting in the production of PS. G3P, glycerol-3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; CDP-DG, cytidine diphosphate diacylglycerol; CMP, cytidine monophosphate; PS, phosphatidylserine; PE, phosphatidylethanolamine.

intermediate

the lack of a structure of the enzyme in complexed with a lipid substrate. Meanwhile, sequence analysis has identified regions at the N and C termini that are abundant in basic amino acids, indicative of potential affinity for the negatively charged surfaces of phospholipid membranes (21, 30). *EcPssA* is categorized as a peripheral membrane protein, with variable cellular localization observed (11, 31). More than 30 years ago, Shibuya (32) postulated a working model wherein the active state of PssA is associated with the inner membrane surface, contributing to PS synthesis, while the cytosolic form is inactive in this synthesis process (33, 34). It has been demonstrated that the protein can detach from the membrane under high-salt concentrations in vitro, whereas its membrane association is promoted by anionic lipids on the membrane surface such as CDP-DG or PG (11, 31). Despite these insights, the molecular determinants



Fig. 2. In vitro assessment of *EcPssA* activity using LC-MS/MS. (A) Comparison of extracted ion chromatogram of 18:1/18:1 PS in the reaction mixture with (black solid line) or without (black dashed line) PssA. (B) Representative MS1 (left) and MS2 (right) spectra of 18:1/18:1 PS from (A). (C) Chemical structure of 18:1/18:1 PS and its potential fragmentation pattern indicated. Calculated mass/charge ratio (*m/z*) values of the precursor and fragment ions are labeled.

governing the enzyme's differential cellular localization remain to be elucidated.

To date, the apo x-ray crystal structure of type I PssA from *H. influenzae* (*Hi*PssA), which exhibits 51% sequence identity with *Ec*PssA, was determined and deposited in the Protein Data Bank (PDB ID: 3HSI) without an accompanying publication. This structure delineates the enzyme in a dimeric form, albeit without any bound ligand, leaving the substrate processing and membrane binding mechanisms unresolved. This study presents two crystal structures of *Ec*PssA: one representing the lipid-free state and the other bound to a substrate CDP-DG, mimicking a Michaelis complex before the formation of the lipid-enzyme covalent intermediate. Our structures represent the monomeric state of the enzyme in contrast to the dimeric form of *Hi*PssA. We further explored the oligomerization state of *Ec*PssA in solution and its relevance to membrane association and enzymatic activity.

RESULTS

Validation of the in vitro activity of recombinant EcPssA

We used liquid chromatography-tandem mass spectrometry (LC-MS/MS) to verify the in vitro synthesis of PS. In the reaction mixture, we confirmed the production of 18:1/18:1 PS with an observed mass/charge ratio (m/z) of [$C_{42}H_{77}NO_{10}P$]⁻ = 786.5244 (calculated m/z = 786.5291) (Fig. 2A), which was further validated by MS/MS analyses (Fig. 2, B and C). Nonspecific reaction products, PA and PG, were also detected in the LC-MS/MS analysis (fig. S2), which presumably originated from the inadvertent incorporation of water or glycerol instead of serine. Similarly, previous studies have reported the ability of *Ec*PssA to use glycerol and *sn*-glycerol-3-phosphate, albeit at slower rates (*21, 24*).

Overall structure of apo EcPssA

We initially attempted to co-crystallize EcPssA with 10:0/10:0 PS, which resulted in crystals diffracting up to a resolution of 2.83 Å

(Table 1). However, no clear electron density corresponding to PS was observed in the final model, leading us to designate the structure as apo PssA (Fig. 3A). The crystallographic asymmetric unit contains 12 molecules of EcPssA (Fig. 3B). However, we were unable to identify a substantial protein-protein interface among protomers, where the calculated interface area ranges from 160 to 490 \AA^2 . Because of the disorder, four to seven residues in the N terminus could not be modeled in all protomers. In addition, electron densities encompassing four to nine residues between $\alpha 5$ and $\alpha 6$ were weak in most subunits. The structure of apo PssA displays a canonical PLDlike fold, comprising two topologically similar domains: the Nterminal domain (residues 27 to 224) and the C-terminal domain (residues 239 to 451) (Fig. 3C). The core of the enzyme consists of two similar β sheets, with seven β strands sandwiched by eight α helices for the N-terminal domain and eight β strands sandwiched by 14 α helices for the C-terminal domain. A pair of His and Lys residues from the conserved HKD motifs is situated symmetrically near the interface between the N- and C-terminal domains (fig. S3A). Specifically, H138 and K140 are located within the β 4- β 5 loop, while H357 and K359 are positioned in the ß11-ß12 loop. In contrast to the HK pairs, D145 and D364 from the HKD motifs are distant from these basic residues. Rather, E385 and D169 are the spatially conserved residues that interact with the corresponding histidine residue in each motif.

Dimer formation of EcPssA in solution

To determine the oligomeric state of *Ec*PssA, we performed sedimentation velocity (SV) experiments with analytical ultracentrifugation (AUC). The AUC results revealed the presence of two dominant species, with approximate sedimentation coefficients of 3.2*S* and 3.9*S* at the 1:1.6 ratio (Fig. 3D). Considering the monomer size of 53 kDa, these species are presumed to correspond to the monomeric and dimeric forms of *Ec*PssA. While the monomers of *Ec*PssA and *Hi*PssA share a high degree of conformational similarity, with root mean square deviation (RMSD) values between 0.71 to 0.96 Å, their Table 1. Data collection and refinement statistics. RMS, root mean square.

	Apo EcPssA (PDB 8YR5)	CDP-DG-bound EcPssA (PDB 8YR6)	
Data collection			
Space group	Р 1	23	
Cell dimensions			
a, b, c (Å)	94.04, 94.10, 194.75	160.50, 160.50, 160.50	
α, β, γ (°)	88.24, 87.70, 60.07	90.00, 90.00, 90.00	
Resolution (Å)	48.64–2.83 (2.88–2.83) [*]	42.89–2.44 (2.54–2.44)	
R _{merge}	0.187 (1.860)	0.256 (5.209)	
R _{pim}	0.088 (0.882)	0.041 (0.836)	
l/σl	8.1 (1.0)	11.0 (1.0)	
Completeness (%)	98.2 (98.0)	100 (100.0)	
Redundancy	5.3 (5.3)	40.5 (39.7)	
Refinement			
Resolution (Å)	48.64–2.83	42.89–2.44	
No. reflections	129010	24360	
R _{work} /R _{free}	0.242/0.285	0.213/0.247	
No. atoms	43895	3779	
Protein	43775	3660	
Ligand/ion	120	69	
Water	0	50	
B-factors (Å ²)			
Protein	71.9	66.2	
Ligand/ion	77.6	83.9	
Water	-	56.1	
RMS deviations			
Bond lengths (Å)	0.008	0.007	
Bond angles (°)	1.527	1.266	

*Values in parentheses are for highest resolution shell.

crystallographic packing is markedly different (fig. S3B). The dimer interface of HiPssA consists of hydrophobic interactions covering an area of 1250 Å², which aligns well with that of *Ec*PssA when superimposed (Fig. 3E). Using AlphaFold, we constructed a model of the EcPssA dimer (Fig. 3F). Using this model, we identified five hydrophobic residues in α21-α22 helices (I316, I441, I443, L446, and I450) and mutated them to Asp to test whether these changes could disrupt dimeric interactions. Analysis of the AUC data revealed that the quintuple mutant behaves like a monomer, suggesting that these residues are critical for the dimerization of EcPssA (Fig. 3D). Furthermore, we attempted to cross-link the monomers by introducing a cysteine mutation at L446, which is positioned at the center of the dimer interface in our model. Using non-denaturing polyacrylamide gel electrophoresis (PAGE), we verified that the L446C mutant was able to form a dimer through a disulfide bond (fig. S3C). These findings support the conclusion that EcPssA maintains an equilibrium between monomer and dimer states, with dimerization mediated by a critical cluster of hydrophobic residues on $\alpha 21$ - $\alpha 22$.

CDP-DG-bound structure

Initial attempts to crystallize the wild-type *Ec*PssA with its substrate CDP-DG were unsuccessful, possibly due to enzymatic turnover of CDP-DG by water or glycerol in the crystallization mixture,

resulting in the lipid dissociating from the enzyme. To capture the conformation of the enzyme in the lipid-bound state, we introduced an alanine mutation at H357, a crucial residue of the HKD motifs for catalytic activity (fig. S4A), and used a less reactive lipid, 16:0/16:0 CDP-DG, which exhibited reduced turnover in our in vitro assays (fig. S4B). These strategies led to the formation of co-crystals with 16:0/16:0 CDP-DG, which diffracted to a resolution of 2.44 Å (Fig. 4A and Table 1), comprising a single EcPssA-16:0/16:0 CDP-DG complex per asymmetric unit. Similar to the apo structure, no strong intermolecular interactions can be observed at the crystallographic interface, thus representing a monomeric state. An overall fold of the lipid-bound form is not substantially different from the apo state, with RMSD values ranging from 0.37 to 0.44 Å between the two structures. However, we observed several changes in local conformation around the 16:0/16:0 CDP-DG binding site. The most notable one is the organization of the loop connecting $\alpha 5$ and $\alpha 6$ upon lipid binding, which is generally disordered in the apo structure (Fig. 4B). Furthermore, the imidazole side chain of H138 shifts by 2.7 Å to interact with the β -phosphate of CDP-DG, accompanied by more subtle shifts in K140 and K359 (fig. S5A). The binding of CDP-DG also prompts the C-terminal L451 side chain to move by ~6 Å, enabling interaction with the lipid's fatty acyl chains (fig. S5B). The carboxyl group of this residue additionally forms a hydrogen bond with

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Fig. 3. Overall conformation of lipid-free *EcPssA*. (A) The overall structure of the *EcPssA* protomer, displaying pseudo-twofold symmetry, is depicted in cartoon representation from two distinct orientations. (B) The molecular arrangement of the *EcPssA* dodecamer within the crystallographic asymmetric unit is illustrated, with individual protein chains distinctly colored. (C) Topology diagram of the apo-*EcPssA*, highlighting the canonical PLD-like domain. The N- and C-terminal domains are differentially shaded for distinction. (D) Analysis of the oligomeric state of wild-type *EcPssA* (top) and quintuple hydrophobic-to-Asp mutant; i.e., I316D/I441D/I443D/ L446D/I450D (bottom) via sedimentation velocity (SV) experiments. *c(s)*, sedimentation coefficient distributions. (E) Crystallographic dimer of *Hi*PssA, with the residues at the dimeric interface displayed as sticks within the dashed box and annotated. (F) The AlphaFold-predicted dimer model of *Ec*PssA. *Ec*PssA residues in the dimerization interface are shown as sticks and labeled.



Fig. 4. Recognition of CDP-DG. (**A**) Overall structure of *Ec*PssA complexed with 16:0/16:0 CDP-DG. The protein is depicted in cartoon representation and the CDP-DG along with the two HK motifs are shown as sticks. Atoms in CDP-DG are colored yellow, red, blue, and orange representing carbon, oxygen, nitrogen, and phosphorous, respectively. (**B**) Comparison of apo and CDP-DG–bound structures. The apo structure is colored gray, while the CDP-DG–bound structure is shown in sky blue. The α 5- α 6 loop becomes organized upon CDP-DG binding. (**C**) Conformation of bound 16:0/16:0 CDP-DG displayed as sticks. The electrostatic potential of *Ec*PssA is represented on the protein surface, with positively and negatively charged surfaces colored blue and red, respectively. A $2F_0$ - F_c map contoured at 1.0 σ surrounding the bound CDP-DG is shown as bright orange mesh. Interactions of *Ec*PssA with (**D**) diphosphate moiety, (**E**) cytidine moiety, or (**F**) diacylglycerol moiety of CDP-DG. Protein residues are colored in teal. Hydrogen bonds and salt bridges are shown as dashed lines.

the backbone of R378 in the lipid-bound structure while maintaining its apo structure hydrogen bonds with Y324 and N376. Notably, these interactions involving the widely conserved C-terminal residue (fig. S6) are crucial for protein stability, as evidenced by aggregate formation in constructs with an affinity tag at the C terminus, which disrupts the α -carboxyl group's presence at this residue.

Recognition of CDP-DG

The lipid-binding site is situated at the juncture of the enzyme's N- and C-terminal domains, where the 16:0/16:0 CDP-DG is seen adopting a distinctively bent conformation around its diphosphate group (Fig. 4C and fig. S7, A and B). This substrate nestles within a niche bridging hydrophobic and hydrophilic cavities at the enzyme's active site, aptly accommodating the diacylglycerol and CDP moieties. The arrangement of the lipid substrate favors an in-line nucleophilic attack by the imidazole ring of H138 on the β -phosphorus atom of CDP-DG, located a mere 2.8 Å from the Ne atom. Notably, the β -phosphate establishes salt bridges with residues H138, K140, and K359 from the HKD motifs of both domains and a hydrogen bond with the side chain of N374 (Fig. 4D). The α -phosphate engages in hydrogen bonding with the side chains of Y159 and Y273 and a salt bridge with R167. Although the H357A mutation precludes direct observation of this residue's wild-type positioning,

superimposition of the wild-type *Ec*PssA apo structure onto the lipid-bound model suggests that the imidazole side chain of H357 would likely form a salt bridge with the α -phosphate at a proximal distance of ~3 Å.

The 2'- and 3'-hydroxyl groups of the cytidine moiety are coordinated by the guanidinium side chain of R91 (Fig. 4E). The cytosine base engages in an extensive hydrogen bonding network with neighboring residues. Specifically, the 2-carbonyl group interacts with the side chain of R94. Both N3 and exocyclic 4-amine groups are coordinated by the main chain of I97. Furthermore, the cytosine base is sandwiched between R96 and F306, forming a guanidinium- π - π stacking interaction (35, 36). Meanwhile, both acyl chains of CDP-DG are positioned within a large hydrophobic surface lined with I97, A133, F306, I316, I317, L320, L323, Y324, L434, L438, 1447, 1450, and L451 (Fig. 4F). C1 through C8 of sn-1 and C1 through C10 of sn-2 acyl chains are buried within the substrate binding channel, while the rest extend into the solvent-exposed protein surface (fig. S8A). Notably, the sn-2 acyl chain is housed in a narrow cavity delineated by $\alpha 15$, $\alpha 21$, and $\alpha 22$, and the *sn*-1 acyl chain is positioned at the edge of $\alpha 15$ and $\alpha 5 - \alpha 6$ loop, making contact with the more solvent-exposed surface of the enzyme. Consequently, the structural analysis reveals comparatively weaker electron densities and higher atomic B factors for the sn-1 acyl chain

than those of the *sn*-2, indicative of a more flexible positioning in the enzyme (fig. S8B).

MD simulation of EcPssA-membrane interaction

To discern the preferential binding orientation of *Ec*PssA within the lipid bilayer, a molecular dynamics (MD) simulation was conducted (Fig. 5A). The initial positioning of *Ec*PssA on the membrane was established using the PPM 3.0 web server (37), setting the stage for a 100-ns MD simulation. The simulations predict a model wherein EcPssA embeds within one leaflet of the bilayer through interactions mediated by its positively charged surfaces (Fig. 5B). During the initial 20 ns, EcPssA slowly rotated on the membrane, submerging its α 21- α 22 helix-break-helix within the membrane. After the initial transition, the protein conserved its global tertiary structure for the remainder of the simulation (Fig. 5A and fig. S9A). The N terminus and the α 5- α 6 loop exhibited notable deviations from their timeaveraged positions, indicating a degree of structural flexibility in these regions (fig. S9B). Notably, the N terminus migrated to the membrane, facilitating hydrophobic interactions via residues M1 and L2, as well as salt bridge formations through positively charged residues K4, K6, R7, and K9, with the membrane phospholipids. This behavior suggests a contributory role of the N terminus in the enzyme's membrane association, some of which are disordered in our structure. Concurrently, the α 5- α 6 loop, initially occluding the active site, reoriented to permit phospholipid access. Of interest, during the simulation, some phospholipids were extracted from the membrane more than 10 Å away from the normal plane of the membrane into the active site of the enzyme (fig. S9C). In the final state of the simulation, EcPssA interacted with the membrane through various regions, including N-terminal loop (M1-K9), coplanar membrane-associated helices ($\alpha 10$, $\alpha 12$, $\alpha 15$, and $\alpha 17$), and a helix-break-helix (α 21 and α 22) (Fig. 5A).

Validation of the membrane association site

Inspection of the structures reveals that clusters of basic amino acid residues are proximal to the CDP-DG binding site on the surface of *Ec*PssA (Fig. 5B). These basic residues are highly conserved, likely indicating their involvement in facilitating interactions with the negatively charged cellular membrane. The conservation of these basic residues suggests their critical role in mediating interactions with the negatively charged cellular membrane. Structural insights propose that the N and C termini, rich in positively charged residues, might act as anchoring points to the membrane (21, 30). The results from the MD simulation allowed us to identify residues within 5 Å from the predicted membrane interface for subsequent site-directed mutagenesis experiments. These residues are mainly located in two groups: patch 1 at the N-terminal (R131, K212, and R219) and patch 2 at the C-terminal domains (K433, R436, R437, R439, R440, R442, R445, and R449) (Fig. 5C). To investigate the electrostatic nature of membrane association, cell lysates expressing recombinant EcPssA were treated with a buffer containing 200 to 700 mM NaCl, and the partitioning between soluble and membrane-bound fractions was assessed via Western blot (Fig. 5, D and E). At a mild salt concentration (200 mM NaCl), ~40% of wild-type EcPssA detached from the membrane. However, this ratio increased to 70% with a higher salt concentration (700 mM NaCl). When the three Arg residues in patch 1 had been replaced with Glu, the triple-mutant protein (3Es) behaved like the wild-type enzyme over the concentration range tested. In contrast, when the eight basic residues in patch 2 were all changed

to Glu, nearly 70% of the octuple-mutant protein (8Es) detached from the membrane at 200 mM NaCl, and increased to complete detachment at 700 mM NaCl. These results align with a model in which membrane association is primarily driven by electrostatic interactions through these basic residues on the reentrant helices α 21 and α 22 and explain the preference of *Ec*PssA for negatively charged lipids over zwitterionic ones.

Reconstitution of membrane binding using liposomes with varying compositions

It is known that PssA prefers the presence of acidic lipids, such as PG or CL, in the membrane for binding (31). To test whether the association of recombinant PssA is affected by the lipid composition of the membrane, we used liposomes with different ratios of PE to PG. These two phospholipids account for ~90% of the total lipid content in the *E. coli* membrane. We adopted a flotation assay to analyze the distribution of EcPssA in the presence of liposomes using a 0 to 40% sucrose gradient (Fig. 5F) (38, 39). When liposomes containing 100% PE and 0% PG (100:0) were used, the majority of PssA was found in the bottom fractions of the gradient, indicating that its binding to the liposome was limited. However, more liposomebound protein was detected in the upper fractions as the PG content increased to 25% (75:25), which is very similar to the natural composition of the *E. coli* membrane. Flotation experiments using liposomes containing 50% (50:50) or 75% (25:75) PG resulted in a similar distribution of PssA, with liposome-bound fractions increasing further compared to the 75:25 sample. These results support previous findings that the membrane association of PssA is promoted by acidic phospholipids. To verify whether EcPssA is active on the liposome, we performed an in vitro assay using the 75:25 liposome containing CDP-DG. The formation of CMP was successfully detected by highperformance liquid chromatography (HPLC) analysis (Fig. 5G), confirming that PssA can access CDP-DG in the lipid bilayer and act on it through membrane association.

Enzyme kinetics of *Ec*PssA variants and implication on catalytic mechanism

To verify the roles of key residues identified in the structure, we conducted kinetic assays on the wild-type and mutant proteins. For a more quantified assessment of the kinetic properties of *Ec*PssA variants, the activities of the enzyme were spectrophotometrically measured by coupling CMP formation to nicotinamide adenine dinucleotide (NADH) oxidation (40-42). We adopted the previously reported surface dilution model to perform quantitative in vitro assays of *Ec*PssA (41). In this model, the enzyme attaches to the micelle composed of a detergent and the lipid substrate and converts CDP-DG and serine to PS and CMP. Optimal activity was observed at a CDP-DG to Triton X-100 ratio of 1:8, a proportion that we subsequently adopted to ascertain the apparent steady-state kinetic parameters (Table 2).

The wild-type protein displayed an apparent k_{cat} ($k_{\text{cat, app}}$) of 69.7 \pm 7.5 s⁻¹ and K_{m} ($K_{\text{m, app}}$) of 0.14 \pm 0.03 mM for 18:1/18:1 CDP-DG measured at 10 mM serine. Next, we investigated the kinetic properties of mutant proteins with mutations in the HKD motifs. Mutants K140S, H357A, and K359A exhibited essentially no activity, with an estimated $k_{\text{cat, app}}$ of less than 0.1 s⁻¹ and unobtainable $K_{\text{m, app}}$. H138A displayed a substantially reduced CMP production rate, leading to a 180-fold decrease in $k_{\text{cat, app}}$ and a by a threefold reduction in $K_{\text{m, app}}$ for 18:1/18:1 CDP-DG. Overall, mutation at residues interacting with the β -phosphate of CDP-DG substantially



Fig. 5. Mechanism of membrane association of *EcPssA***. (A) A representative molecular dynamics (MD) simulation snapshot at 100 ns showing membrane-bound apo** *EcPssA***. The protein is depicted as a sky-blue cartoon, and the phospholipid membrane is represented in yellow and orange sticks. Cyto, cytoplasm; IM, inner membrane; Peri, periplasm. (B) The electrostatic potential of CDP-DG-bound** *EcPssA* **displayed on the protein surface. (C) Clusters of positively charged residues, which are predicted to interact with the membrane, are highlighted in cyan sticks. (D and E) Membrane association assays for** *EcPssA* **wild-type (WT) and positive-to-negative charge mutants (3Es and 8Es) under varying salt concentration. (D) Representative Western blots illustrating the partitioning of** *EcPssA* **between membrane-bound (Mem) and soluble (Sol) fractions. (E) Quantification of the average band intensities from the Western blots, with error bars representing standard deviations from three independent experiments. (F) Schematic representation of flotation assay. A mixture containing liposomes (composed of PE and PG at ratios of 100:0, 75.25, 50:50, and 25:75) and** *EcPssA* **was incubated for 1 hour and then subjected to sucrose gradient centrifugation to isolate the floated fraction. The gradient was divided into seven layers, and the protein content from each layer was subsequently analyzed by Western blot. (G) High-performance liquid chromatography (HPLC) analysis of** *EcPssA* **WT activity in liposomes (PE:PG = 75:25) embedded with CDP-DG, monitoring CMP production at 278 nm. The solid black line indicates the reaction when PssA is present, while the black dashed line represents the negative control with no enzyme. mAU, milli-Absorbance Units.**

Table 2. Michaelis-Menten parameters for EcPssA WT and mutants. For the MM parameters of 18:1/18:1 CDP-DG, apparent values are reported. The lipid and detergent Triton X-100 were kept in 1:8 ratio. L-serine (10 mM) was used. For the MM parameters of L-serine, 0.3 mM of 18:1/18:1 CDP-DG and 2.4 mM Triton X-100 were used. N.D., not determined due to low activity.

Constructs	k _{cat, app} (/s)	K _{m, app} (mM)	k _{cat} /K _m (/s/mM)	Ligand interactions
18:1/18:1 CDP-DG				
WT	69.7 ± 7.5	0.14 ± 0.03	510	
H138A	0.38 ± 0.02	0.048 ± 0.010	7.9	β-Phosphate
K140A	<0.1	N.D.	N.D.	β-Phosphate
H357A	<0.1	N.D.	N.D.	β-Phosphate
K359A	<0.1	N.D.	N.D.	β-Phosphate
Y57A	43.8 ± 5.8	0.15 ± 0.04	290	Near ribose
R91A	0.97 ± 0.067	0.0092 ± 0.0082	105	Ribose
R94A	1.15 ± 0.03	0.0086 ± 0.0040	133	Cytosine base
R96A	62.3 ± 5.8	0.12 ± 0.03	510	Cytosine base
Y159A	7.0 ± 0.6	0.051 ± 0.014	140	α-Phosphate
R167A	0.33 ± 0.02	0.033 ± 0.005	10	α-Phosphate
Y273A	0.42 ± 0.03	0.051 ± 0.005	8	α-Phosphate
D305A	0.23 ± 0.02	0.021 ± 0.006	11	Near cytosine base
F306A	3.0 ± 0.1	0.012 ± 0.004	240	Cytosine base
L-serine				
WT	59.8 ± 1.7	0.35 ± 0.03	170	
Y57A	23.1 ± 1.4	1.8 ± 0.2	13	Carboxyl group
R91A	1.73 ± 0.1	0.11 ± 0.03	16	Carboxyl group
Y159A	43.0 ± 3.2	0.37 ± 0.1	120	Hydroxyl side chain
R167A	0.48 ± 0.02	0.11 ± 0.02	4	Hydroxyl side chain
Y273A	0.77 ± 0.12	0.83 ± 0.38	1	Amino group
D305A	0.58 ± 0.08	0.61 ± 0.28	1	Amino group

reduced the $k_{cat, app}/K_{m, app}$ ratio by at least 60-fold. Attempts to generate alanine-substituted mutants for D145 and D364, which are part of the conserved HKD motifs, as well as for D169 and E385 that coordinate with the histidine residues within the HKD motifs (see fig. S5A), were unsuccessful. These mutant proteins demonstrated poor stability during purification steps and were not suitable for our assay.

Other amino acid residues involved in polar/ionic interactions with various parts of CDP-DG in the structure were selected for site-directed mutagenesis and subsequent kinetic analyses. Mutants Y159A, R167A, and Y273A, which interact with the α -phosphate of CDP-DG, displayed substantially decreased $k_{cat, app}$ and showed an ~10-fold reduction in $K_{m, app}$ compared to the wild-type. Similarly, the alanine mutants of R91, R94, D305, and F306, which are positioned around the ribose or nucleobase of CDP-DG, resulted in substantially reduced $k_{cat, app}$. In contrast, Y57A and R96A displayed kinetic properties comparable to those of the wild-type. Notably, the side chain of Y57 is stacked above the ribose ring and R96 interacts with the cytosine base of the lipid substrate by a guanidinium– π - π stacking interaction together with F306 in the structure.

In addition, $k_{\text{cat, app}}$ and $K_{\text{m, app}}$ of *Ec*PssA were measured at various concentrations of L-serine while maintaining the concentration of 18:1/18:1 CDP-DG at 0.3 mM. This is necessary as concentrations higher than 0.3 mM resulted in instability of the coupling enzymes. For the wild-type enzyme, $k_{\text{cat, app}}$ of 59.8 \pm 1.7 s⁻¹ and $K_{\text{m, app}}$ of

 0.35 ± 0.03 mM were obtained. Given that the serine-bound structure of PssA is unavailable, we constructed a model by docking a serine molecule onto the active site of the phosphatidyl-histidine covalent intermediate derived from the CDP-DG-bound structure (Fig. 6A). The serine-docked model indicates that the hydroxyl group of serine is well positioned for an in-line nucleophilic attack on the phosphorous center of the enzyme-lipid intermediate. In the model, the side chain of serine forms multiple hydrogen bonds with H357, Y159, and the phosphoramidate oxygen atoms. Notably, the α -amino group of serine forms a salt-bridge with D305 and a hydrogen bond with Y273, whereas the α -carboxyl group interacts with R91 and Y57 via a salt-bridge and hydrogen bond, respectively. Subsequently, we introduced mutations into the residues potentially interacting with serine, as predicted by our docking model, and investigated the kinetic properties of these mutant proteins. The alanine mutant of H357 exhibited essentially no activity under our assay conditions as expected (fig. S10). While the alanine mutants of R91, R167, Y273, and D305 displayed a reduced CMP production rate, leading to a 30- to 100-fold decrease in $k_{\text{cat, app}}$ because these residues appear to participate in the interactions with CDP-DG as well. The $K_{m, app}$ of Y273A and D305A increased by approximately twofold, supporting our docking model that these residues are interacting with the α -amino group of serine. Notably, the Y57A mutant exhibited a $k_{\text{cat, app}}$ of Y57A decrease by approximately twofold and a more than fivefold increase in $K_{m, app}$. Given that this variant



Fig. 6. A proposed model for cellular localization and catalysis of *EcPssA*. (A) The active site of a serine-docked model of *EcPssA* with phosphatidic acid conjugated at H138. Protein side chains and ligands are colored in cyan and yellow, respectively. Hydrogen bonds and salt bridges are shown as dashed lines. (B) A hypothesis illustrating the dimerization and membrane association of cytosolic *EcPssA*. *EcPssA* exists in a dimer-monomer equilibrium in the cytosol but the monomeric form only can bind to the membrane because the dimerization interface substantially overlaps with the membrane-binding surface of the protein. Cyto, cytoplasm; IM, bacterial inner membrane; Peri, periplasm. (C) A proposed catalytic mechanism of *EcPssA*. The nucleophilic attack of H138 on the phosphorous of the CDP-DG releases the CMP group and forms a phosphatidyl-histidyl intermediate. Subsequently, activation of serine by H357 enables nucleophilic attack on the phosphorous to yield PS. R denotes diacylglycerol moiety.

maintained nearly wild-type kinetics in the presence of varying CDP-DG concentrations at a saturating serine level, Y57 appears to play a substantial role in serine recognition and binding. Mean-while, kinetic parameters for Y159A were not notably different from the wild-type for serine, in contrast to the 10-fold reduction of $k_{\text{cat, app}}$ for CDP-DG. This indicates that Y159 is more intimately involved in CDP-DG binding rather than in serine interaction.

DISCUSSION

Oligomerization-dependent cellular localization of EcPssA

It has been shown that *Ec*PssA exists in two states: a soluble latent state and a membrane-bound active state. While the cytosolic PssA co-purifies with ribosome, the physiological significance of the ribosome binding is unknown. Conversely, its association with the membrane is enhanced by the presence of negatively charged phospholipids. Despite the long-standing hypothesis regarding this regulatory mechanism, detailed molecular insights into the individual states have been elusive. Our analysis of AUC results for *Ec*PssA variants indicates that the cytosolic enzyme exists in a monomer-dimer equilibrium, with only the monomeric form capable of binding to the bacterial membrane (Fig. 6B). While previously reported structure of PssA from H. influenzae reveals a dimeric form, our structures show the monomeric state. In the dimer model of EcPssA, the dimer interface is composed of multiple hydrophobic residues from one side of the amphipathic helix, which simultaneously engages in membrane association through clusters of basic residues on another side. Upon membrane binding, the hydrophobic residues essential for dimerization become available to facilitate the docking of the lipid substrate. Therefore, we hypothesize that the membrane association and dimerization of PssA are mutually exclusive events, allowing only the monomeric form to bind to the membrane. The oligomerization of cytosolic PssA adds an additional layer of the regulatory complexity, likely related to the balancing of the level of phospholipids. Notably, CDP-DG serves as a precursor for the synthesis of PG: PgsA assembles PG phosphate (PGP) from PG and glycerol 3-phosphate, while PgpABC remove the phosphate from PGP to yield PG. Some of the PG is further converted to CL by ClsABC. Discovering factors that influence oligomerization and the biological functions of cytosolic PssA will

enhance our understanding of oligomerization-driven regulation of phospholipid homeostasis in bacteria.

Catalytic mechanism of EcPssA

While previous mutagenesis experiments have underscored the significance of the dual HKD motifs in PssA function (25), the exact contributions of individual residues within these motifs have been ambiguous. Specifically, the identity of the histidine residue implicated in forming a covalent intermediate with CDP-DG has been elusive. Our lipid-bound structure of EcPssA, containing intact CDP-DG, decisively reveals the precise interactions between the enzyme and the lipid. H138 is proximal to the β-phosphate of CDP-DG, positioning it optimally for nucleophilic attack. Because this complex was formed using the catalytically inactive H357A mutant enzyme, H357 is posited to be instrumental for lipid accommodation and covalent intermediate formation. Both K140 and K359 form salt bridges with the β -phosphate, while the aspartate residues from conserved HKD motifs, D145 and D364, are remotely located from the active site suggesting their catalytic insignificance. Instead, E385 and D169 are posited as the operative participants in the HKD motif's functional repertoire. These observations imply that PssA may use a distinct strategy diverging subtly from the canonical pathways of the PLD superfamily.

Unlike PLD, PssA resolves the enzyme-phospholipid intermediate with serine instead of water. The precise binding mode of serine, however, remains mysterious due to the absence of a complex structure. In our serine-docked model, the α -amino group of serine is predicted to form a salt bridge with the D305, a residue found to be pivotal for catalytic function through mutagenesis experiments. The remote location of D305 suggests that the inactivity of D305A may arise primarily from compromised serine interaction. In addition, within the context of our model, R91 and R96 emerged as candidates for interaction with serine's carboxylate due to their spatial proximity. Considering that the R96A variant retains wild-type-like activity, R91 is posited as the more probable contributor to serine binding via salt bridge formation. The serine hydroxyl group is situated within a sterically hindered environment, possibly making contact with several residues, including H357, Y159, and a phosphoramidate linked to H138. This constricted space near the phosphorus atom may permit smaller nucleophiles such as water or glycerol to access the active site, although possibly at a reduced reaction rate for the latter. This theory might elucidate the observed low reactivity of threonine with PssA, given its bulkier secondary alcohol group. The relatively high pK_a (where K_a is the acid dissociation constant) for the serine side chain implies the necessity for a general base to prime this, otherwise, weak nucleophile. Y159, although proximate to the hydroxyl group of serine, is disqualified as a candidate based on kinetic studies, as the Y159A mutant sustains activity. Consequently, we hypothesize that H357 serves as the general base, with its function reinforced by its interaction with D169, akin to the manner in which E385 bolsters the nucleophilicity of H138 for covalent intermediate formation in the previous step (Fig. 6C). The arrangement of D169-H357-serine in PssA resembles the classic catalytic triad (D/E-H-S) found in serine proteases, where serine residue acts as the nucleophile. Nonetheless, alternative mechanisms influencing serine deprotonation cannot be dismissed and warrant further examination to clarify the precise enzymatic process.

MATERIALS AND METHODS

Cloning, protein expression, and purification

The pssA gene was amplified from the genomic DNA of E. coli strain MG1655 by polymerase chain reaction using primers listed in table S1 and inserted into a plasmid vector (pLATE31, Thermo Fisher Scientific) featuring an N-terminal His6-Tag sequence, using the method provided by the manufacturer. Site-directed mutant plasmids were generated using in vivo cloning (43). The DNA sequences were confirmed by Macrogen (Korea). For protein expression, E. coli BL21(DE3) cells transformed with the expression vector harboring wild-type or mutant pssA gene were grown in Luria-Bertani (LB) medium containing ampicillin (100 µg/ml) at 37°C with shaking at 160 rpm for aeration until the optical density at 600 nm (OD_{600}) reached 0.4 to 0.8, while the L446C mutant was specifically expressed in E. coli SHuffle T7 express (New England Biolabs Inc.) under the same conditions. Protein expression was induced by adding 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) to the culture, which was further incubated for an additional 3 hours. The cells were then harvested by centrifugation at 14,372 rcf (9000 rpm) and 4°C for 10 min, washed with buffer A [50 mM tris-HCl (pH 7.5), 500 mM NaCl, and 10% (v/v) glycerol], and pelleted at 3214 rcf (3900 rpm) and 4°C for 30 min. The cells were resuspended in buffer A containing 50 mM imidazole, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, and lysozyme (1 mg/ml) and incubated on a threedimensional rocker at 20°C for 20 min. The cells were then lysed by sonication for 10 cycles of 5-s pulse and 25-s rest intervals at 50% amplitude, and the membrane protein was solubilized by incubating the lysate with 1% (w/v) octyl β -D-glucopyranoside (OG) (Carbosynth) at 4°C for 1 hour with rocking. The detergent-added lysate was centrifuged at 21,672 rcf (14,000 rpm) and 4°C for 60 to 120 min, filtered through a 0.2-µm-pore size syringe filter, and applied to a HisTrap HP 5-ml column (GE Healthcare) charged with Ni²⁺ and pre-equilibrated with buffer A supplemented with 50 mM imidazole and 0.1% (w/v) OG. After the elution with an imidazole concentration gradient up to 500 mM, the protein was further purified by size exclusion chromatography using a HiLoad 16/600 Superdex 200-pg column (GE Healthcare) equilibrated with buffer B [30 mM tris-HCl (pH 7.5), 1 M NaCl, 10% (v/v) glycerol, and 0.05% (w/v) OG]. The pooled fractions were buffer exchanged with buffer C [30 mM tris-HCl (pH 7.5), 400 mM NaCl, 10% (v/v) glycerol, and 0.05% (w/v) OG]. The purity of the protein was assessed using SDS-PAGE analysis, and the yield was determined using the extinction coefficient calculated by the ProtParam tool ($\varepsilon_{280} = 68300 \text{ M}^{-1} \text{ cm}^{-1}$). The purified protein was concentrated using a 50-kDa-molecular weight cutoff 15- or 0.5-ml Amicon Ultra centrifugal filter (Millipore).

LC-MS analysis of functional activity

The assay condition developed by Dowhan (44) was adopted for our assays with minor modifications. In brief, the assay solution was prepared by mixing 0.4 mM 18:1/18:1 CDP-DG (Avanti Polar Lipids), 1 mM L-serine, and bovine serum albumin (BSA; 1 mg/ml). The reaction was initiated by adding 20 μ M *EcPssA* in buffer C supplemented with 0.5 mM DTT and incubated at 37°C for 30 min. The reaction was quenched by transferring the assay solution to a glass tube containing 5 volumes of the chloroform/methanol (2:1, v/v) mixture. Subsequently, lipids in the reaction samples were extracted by the Folch method (45). The tube was briefly vortexed and centrifuged at 500 rcf and 4°C for 10 min. The lower phase was collected in a glass tube, dried out, and dissolved in methanol for LC-MS

analysis. An aliquot of 20 µl was injected into a reversed-phase HPLC column (ZORBAX RR Eclipse Plus C18, 95 Å, 4.6 mm by 100 mm, 3.5 µm; Agilent Technologies) attached to Agilent 1260 Infinity Quaternary LC system (Agilent Technologies). Chromatographic separation was performed by using a gradient step composed of solvent A (10 mM ammonium acetate in water) and solvent B (10 mM ammonium acetate in isopropyl alcohol) with the flow rate of 0.5 ml/ min as follows: (i) 0% B for 3 min; (ii) a linear gradient from 0 to 60% B for 2 min; (iii) a linear gradient from 60 to 100% B for 4 min; (iv) a hold at 100% B for 5 min; (v) a linear gradient from 100 to 0% B for 1 min; and (vi) a hold at 0% B for 5 min. Eluted samples were analyzed by LC-MS and LC-MS/MS (Agilent 6520 Q-TOF LC/MS; Agilent Technologies) in negative mode using Dual ESI as an ionization source. Major phospholipids were identified and relatively quantified using the MassHunter program with the aid of Chem-Draw. Results were visualized by OriginPro 2021 (OriginLab).

Crystallization of EcPssA Apo EcPssA

The protein sample of wild-type EcPssA was mixed with 10:0/10:0 PS (Avanti Polar Lipids) to final concentrations of 0.093 mM protein and 1 mM lipid in buffer C. Crystallization was performed via sitting drop vapor diffusion methods at 20°C by mixing 0.8 µl of protein solution with 0.8 µl of reservoir solution equilibrating against 60 µl of the reservoir solution containing 0.09 M sodium citrate tribasic dihydrate (pH 5.9), 0.09 M lithium sulfate monohydrate, 0.1 M sodium malonate (pH 7.0), and 12.6% (w/v) polyethylene glycol 4000. Hexagon-shaped crystals were formed within 1 week. The crystals were cryo-protected by mother liquor supplemented with 20% (w/v) polyethylene glycol 4000 before flash freezing in liquid nitrogen.

16:0/16:0 CDP-DG bound-EcPssA

For the crystallization of EcPssA H357A-16:0/16:0 CDP-DG, the protein solution of H357A EcPssA was mixed with 16:0/16:0 CDP-DG (Avanti Polar Lipids) to final concentrations of 0.093 mM EcPssA H357A and 0.56 mM 16:0/16:0 CDP-DG in buffer C. Cubic-shaped crystals were obtained by hanging drop vapor diffusion method at 20°C by mixing a 1.5 µl of protein solution with 1.5 µl of reservoir solution equilibrating against 500 µl of the mother liquor containing 0.1 M lithium sulfate monohydrate, 0.1 M ADA (pH 6.5), 12% polyethylene glycol, 2% (w/v) 2-propanol, and 0.5% (w/v) n-dodecyl-β-D-maltopyranoside within 1 week. The crystals were flash freezing in liquid nitrogen without cryo-protectant.

Data acquisition and processing

The x-ray diffraction data were collected under cryogenic conditions using a wavelength of 1.000 Å at the Pohang Accelerator Laboratory beamline 5C in Korea, equipped with an Eiger X 9M detector. The diffraction data from a single apo crystal were integrated by autoPROC (46), scaled by XSCALE in XDS (47) suite, and merged by Aimless (48) in CCP4 (49) suite. The data from a single CDP-DG complexed crystal were integrated by DIALS (50) pipeline in Xia2 (51) and then scaled and merged by Aimless (48) in CCP4 Cloud (52) suite. The phase determination of the structure was performed using MolRep (53) with AlphaFold predicted structure (accession code AF-P23830-F1) (54) as a search model. Iterative real space model building and refinements were carried out with Coot (55), Refmac5 (56), and Phenix.refine (57), with the aid of PDB_REDO (58), ModelCraft (59), and lorestr (60). The resulting model was visualized with PyMOL

(61) and Protein Imager (62). Ramachandran statistics are 95.25% favored, 4.75% allowed, and 0.00% outliers for apo and 96.38% favored, 3.62% allowed, and 0.00% outliers for CDP-DG complexed structures.

Analytical ultracentrifugation

SV experiments were performed in Optima AUC (Beckman Coulter, USA) using An-60 Ti rotor using the following conditions: rotation speed, 42,000 rpm; number of scans, 150 to 160; scan interval, 3 min; and temperature, 20°C. A EcPssA wild-type protein (0.6 to 0.8 mg/ ml) dissolved in buffer C excluding glycerol was loaded into a 12-mm two-sector EPON centerpiece and quartz window filled with a 400-µl sample. Samples were allowed to equilibrate for 1 hour at

Too construct a full-length wild-type *Ec*PssA model, we used the CDP-DG complexed structure as a template. The unobserved N terminus was reconstructed on the basis of the AlphaFold2 (54)– predicted model, and the H357A mutation was reverted to His. To mimic an apo state, the bound CDP-DG was removed. The initial positioning of *Ec*PssA within the membrane was determined using the positioning of proteins in membranes (PPM3.0) server (37). The inner membrane of Gram-negative bacteria (64) surrounding the protein was generated using the CHARMM-GUI membrane builder protein was generated using the CHARMM-GUI membrane builder tool v3.7 (65), with 190 phospholipids in the outer leaflet and 174 in the inner leaflet. Detailed phospholipids composition is available in table S2. A simulation box with dimensions of 110 Å by 110 Å by 126 Å was neutralized by adding 158 K⁺ ions and 83 Cl⁻ ions to achieve a 0.15 M ionic strength. Subsequently, the neutralized system was solvated with 29,758 TIP3 water molecules, resulting in a total of 142,317 atoms. MD simulations were conducted using the OpenMM (66) engine with the CHARMM36m force field within a Jupyter Notebook hosted on Google Colab (67). The system was maintained at a constant temperature of 303.15 K and pressure of 1 bar throughout the simulations using a Langevin thermostat and a Monte Carlo barostat, respectively. The system was minimized for 1000 steps, followed by an equilibration for 2 ns with a time step of 2 fs. During the equilibration, the protein and membrane were constrained by a constant force of 800 kJ/mol. After the equilibration, triplicates of unrestrained production simulations were run for 100 ns. The trajectory file was generated every 100 ps, and the resulting trajectory was aligned and analyzed using MDAnalysis (68, 69).

Preparation of serine docking model

To generate a PA-conjugated model, we used the CDP-DG complex structure as a template. The CMP moiety was deleted. Then, a link connecting the phosphorous atom of the PA and NE of H138 was generated in Coot (55). To apply restraints to the PA-conjugated model, the model was refined with phenix.refine (57). After the refinement, a serine molecule was rigid-body docked on the model using HDOCK (70).

Membrane association assay

E. coli BL21(DE3) cells transformed with the expressing plasmid for the wild-type EcPssA or glutamate mutants were incubated in 1 liter of LB medium supplemented with ampicillin (100 μ g/ml). When OD_{600} reached ~0.4, the protein expression was induced by the addition of IPTG to a final concentration of 1 mM. The flask containing the cells was incubated at 37°C for an additional 3 to 4 hours with shaking at 160 rpm. The cells were harvested by centrifuge at 14,372 rcf (9000 rpm) and 4°C for 10 min, resuspended by 1× phosphate-buffered saline (PBS) [3 mM Na₂HPO₄, 1.1 mM KH₂PO₄, and 160 mM NaCl (pH 7.4); LPS Solution, Korea], and pelleted at 3214 rcf and 4°C for 30 min. The cell pellets were stored at -86°C until used. The cell pellets resuspended in 30 ml of $1 \times PBS$ were lysed by sonication. The lysates were aliquoted; diluted with a buffer containing 200, 300, 400, 500, 600, or 700 mM NaCl; and incubated at 4°C for 1 hour. After separating the inclusion bodies by centrifugation at 21,672 rcf (14,000 rpm) and 4°C for 30 min, the supernatant was subjected to ultracentrifugation at 120,000 rcf (35,000 rpm) and 4°C for 1 hour, which was equipped with 8× 13.5 ml of rotor and Ultra 5.0 (Hanil Scientific, Korea). After sedimentation, the supernatant (soluble fraction) and the pellet (membrane fraction) were collected separately. The pellet was lysed in 1× PBS supplemented with 0.1% SDS. Each fraction was analyzed by 12% SDS-PAGE and visualized by Western blotting using horseradish peroxidase (HRP) Anti-6X His tag antibody (Abcam, UK).

Spectrophotometric assays for measuring CMP formation rate

Phosphoenolpyruvate (PEP), adenosine 5'-triphosphate (ATP), and BSA were purchased from Sigma-Aldrich. L-serine was purchased from Alfa Aesar. NADH, pyruvate kinase (PK), and lactate dehydrogenase (LDH) were purchased from Roche. CMP kinase (CMPK) was purchased from Megazyme. Triton X-100 was purchased from Daejung, Korea. CDP-DG (18:1/18:1; catalog no. 870520P) was purchased from Avanti Polar Lipids.

PS synthase activity was determined by measuring the release of CMP from 18:1/18:1 CDP-DG at 25°C by following the decrease in absorbance at 340 nm on Eppendorf BioSpectrometer as described by Carman and Dowhan (40, 41). The reaction mixture contained 50 mM tris-HCl (pH 7.5), 0.1 M KCl, 10 mM MgCl₂, BSA (1 mg/ml), 10 mM L-serine, 1 mM ATP, 1 mM PEP, 0.2 mM NADH, CMPK (0.2 U/ml), PK (0.4 U/ml), LDH (0.4 U/ml), and varying concentrations of Triton X-100 and 18:1/18:1 CDP-DG substrate in a total volume of 0.18 ml, where the assay was initiated by adding *Ec*PssA. The sample was briefly sonicated before absorbance measurements to ensure proper homogenization.

Preparation of liposomes

The phospholipids 16:0/18:1 PE (Avanti Polar Lipids), 16:0/18:1 PG (Avanti Polar Lipids), and 18:1/18:1 CDP-DG (Avanti Polar Lipids) were dissolved in chloroform and mixed in varying proportions. For the flotation assay, lipid mixtures were prepared by combining PE and PG at molar ratios of 100:0, 75:25, 50:50, and 25:75, respectively. For the activity test, a lipid mixture was prepared by combining PE, PG, and CDP-DG at a molar ratio of 6:2:1. The solvent was then evaporated to obtain a thin lipid film. The lipid film was hydrated in buffer C at room temperature for 1 hour. Unilamellar vesicles were then prepared by extruding the multilamellar vesicles 19 times through a 0.1- μ m polycarbonate filter using a miniextruder (Avanti Polar Lipids).

Flotation assay

For protein binding to liposome membranes, *Ec*PssA WT (1 mg/ml) in buffer C was incubated with liposomes (2 mM; varying proportions

of PE:PG) for 1 hour at a protein-to-lipid molar ratio of 1:100. To verify protein association to the liposomes, a sucrose gradient ranging from 0% (w/v) at the top to 40% (w/v) at the bottom was prepared as follows: Proteoliposomes were mixed with 80% (w/v) sucrose in buffer C to achieve a final concentration of 40% (w/v) sucrose (bottom layer). A 25% (w/v) sucrose solution in buffer C was used for the middle layer, and buffer C alone was used for the top layer. The established gradient was centrifuged at 180,000 rcf at 20°C for 2 hours using an Optima MAX-XP ultracentrifuge with a TLS-55 rotor (Beckman Coulter). Top and bottom fractions were collected manually, analyzed by 12% SDS-PAGE, and visualized using Western blotting with HRP Anti-6X His tag antibody (Abcam, UK).

Supplementary Materials

The PDF file includes: Figs. S1 to S10 Table S2 Legend for table S1

Other Supplementary Material for this manuscript includes the following: Table S1

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