Structural insights into phosphatidylethanolamine formation in bacterial membrane biogenesis

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Phosphatidylethanolamine (PE), a major component of the cellular membrane across all domains of life, is synthesized exclusively by membrane-anchored phosphatidylserine decarboxylase (PSD) in most bacteria. The enzyme undergoes auto-cleavage for activation and utilizes the pyruvoyl moiety to form a Schiff base intermediate with PS to facilitate decarboxylation. However, the structural basis for self-maturation, PS binding, and decarboxylation processes directed by PSD remain unclear. Here, we present X-ray crystal structures of PSD from *Escherichia coli*, representing an apo form and a PE-bound complex, in which the phospholipid is chemically conjugated to the essential pyruvoyl residue, mimicking the Schiff base intermediate. The high-resolution structures of PE-complexed PSD clearly illustrate extensive hydrophobic interactions with the fatty acyl chains of the phospholipid, providing insights into the broad specificity of the enzyme over a wide range of cellular PS. Furthermore, these structures strongly advocate the unique topology of the enzyme in a lipid bilayer environment, where the enzyme associates with cell membranes in a monotopic fashion via the N-terminal domain composed of three amphipathic helices. Lastly, mutagenesis analyses reveal that *E. coli* PSD primarily employs D90/D142–H144–S254 to achieve auto-cleavage for the proenzyme maturation, where D90 and D142 act in complementary to each other.
of the N-terminus serine to pyruvoyl in the α-subunit, which is an indispensable prosthetic group for decarboxylation activity\(^2\). PSD is a serine protease that employs a classic catalytic triad composed of Asp-His-Ser, where nucleophilic serine attacks the scissile peptide bond to produce α- and β-chains\(^2\). Through mutagenesis experiments, D139-H198-S308 and D210-H345-S463 were identified as the catalytic triads for PSD in Plasmodium knowlesi\(^2\) and *S. cerevisiae*\(^2\), respectively. After the proenzyme undergoes auto-proteolysis, an active complex is formed between the α- and β-chains, which are mainly responsible for catalytic activity and membrane association, respectively. The reaction mechanism for PSD was proposed based on that of pyruvoyl-dependent histidine decarboxylase\(^2\), where the amine group of PS and the α-carbonyl carbon of pyruvoyl form a Schiff base intermediate, followed by decarboxylation\(^2\).

The first crystal structures of PSD from *E. coli* in apo- and lipid-bound forms have been reported recently, which offer valuable information regarding the membrane-associated mechanism, substrate binding, and determinants critical for catalytic activity\(^2\). The lipid-bound structure displays covalent conjugation of PE to the pyruvoyl residue; however, a rather low resolution (3.60 Å) limits the identification of acyl chain moieties of the bound lipid molecule. Here, we report two crystal structures of *E. coli* PSD, representing apo states at resolutions of 1.90 and 2.63 Å, along with two PE-bound structures of PSD at resolutions of 2.12 and 2.70 Å, which mimic the Schiff base intermediate formed between the pyruvoyl group and a phospholipid. Our high-resolution structures allow the identification of the exact locations where intermolecular interactions occur between the enzyme and the bound phospholipid molecule via diacyl chains in particular. Structure-guided mutagenesis analyses confirmed the key residues involved in phospholipid recognition, decarboxylation of PS, and maturation of PSD. In particular, we identified the crucial residues required for the activation of proenzymes, which have not been characterized for *E. coli* PSD.

**Results**

To facilitate crystallization and structure determination, we employed a recombinant *E. coli* PSD with the last 35 residues removed (PSD\(_{1-287}\)). The amino acid sequences of this region are missing in most bacteria, except for certain species of Enterobacterales (Supplementary Fig. S1). The in vitro activity of PSD\(_{1-287}\) was tested using soy PS as a substrate, and five major phospholipid species were quantitatively analyzed via LC–MS (Fig. 1B and Supplementary Fig. S2A). Under our assay conditions, it is estimated that over 95% of PS is enzymatically converted to PE. This result is comparable to that of the full-length wild-type enzyme (Supplementary Fig. S2B), indicating that truncation of the C-terminal residues does not significantly contribute to decarboxylation activity.

**Overall feature of apo structures.** Using native and selenomethionine-derivatized recombinant PSD\(_{1-287}\), the crystal structures of apo PSD\(_{1-287}\) were determined from two crystal forms at resolutions of 1.90 and 2.63 Å, which are described as Apo-PSD1 and Apo-PSD2, respectively. The crystallographic statistics are summarized in Table 1. The asymmetric unit of Apo-PSD1 contains two αβ-heterodimers, whereas that of Apo-PSD2 contains four (Supplementary Fig. S3). The biological assembly of PSD\(_{1-287}\) appears to be a dimer of αβ-heterodimers, (αβ)\(_2\), which is commonly identified in all four structures presented herein (Fig. 2A). The heterotetrameric form of PSD\(_{1-287}\) in solution was consistent with the results of the SEC-MALS analysis (Supplementary Fig. S4). Two out of three molecules of N-dodecyl-b-maltoside (DDM), a detergent used to solubilize the enzyme, were located at the interface between two adjacent β-subunits, augmenting the dimerization of αβ-heterodimers in Apo-PSD1. No detergent molecules were modeled in Apo-PSD2 structure. The α and β subunits form a tight 1:1 complex in each heterodimer with an average interface area of 1790 Å\(^2\), where 29 out of 34 residues from the α subunit participate in forming the dimerization interface. Using the N-terminal sheet composed of residues from Pvl-254 to Ala-261, the α-subunit was integrated between two anti-parallel sheets of the β-subunit.
Identification of the membrane association domain. The three N-terminal helices contributed by each β-subunit lie in a plane, where hydrophobic residues are aligned on one side of the helices (Fig. 2A). To investigate the role of the three helices in membrane association, the cellular location of the wild-type protein and a series of truncated PSD in the N-terminal domain were examined via immunoblotting of soluble, membrane, and insoluble fractions after cell lysis (Fig. 3B). The mutant ΔH1 lacks only the N-terminal helix 1, whereas ΔH2 lacks helices 1 and 2, and ΔH3 is devoid of helices 1–3. Most wild-type proteins were detected in the membrane fraction, whereas ΔH1 exhibited enrichment in the soluble fraction, as well as in the membrane fraction. Additional truncations led to incomplete auto-cleavage of the proenzyme in

Table 1. Crystallographic data collection and refinement statistics. Each dataset was collected from a single crystal. Values in parentheses are for highest-resolution shell.

| Data collection | Apo-PSD1 (PDB: 7CNW) | Apo-PSD2 (PDB: 7CNX) | 8PE-PSD (PDB: 7CNY) | 10PE-PSD (PDB: 7CNZ) | Apo-PSD1 (SeMet) |
|-----------------|-----------------------|-----------------------|----------------------|----------------------|------------------|
| Wavelength (Å)  | 0.9801                | 0.9793                | 0.9796               | 0.9796               | 0.9793           |
| Space group     | P212121               | P212121               | P212121              | P212121              | P212121          |
| Cell dimensions |                       |                       |                      |                      |                  |
| a, b, c (Å)     | 77.4, 79.82, 147.09   | 79.90, 101.85, 170.10 | 77.15, 79.46, 146.77 | 79.72, 102.37, 168.99 | 77.35, 78.73, 147.46 |
| a, β, γ (°)     | 90.00, 90.00, 90.00   | 90.00, 90.00, 90.00   | 90.00, 90.00, 90.00  | 90.00, 90.00, 90.00  | 90.00, 90.00, 90.00 |
| Resolution (Å)  | 49.03–1.90 (1.94–1.90) | 48.78–2.63 (2.73–2.63) | 29.35–2.12 (2.82–2.70) | 29.47–2.70 (2.18–2.12) | 34.73–2.12 (2.18–2.12) |
| R cryst | 0.132 (2.802) | 0.146 (2.609) | 0.122 (2.231) | 0.185 (2.649) | 0.267 (3.540) |
| R free | 0.037 (0.845) | 0.055 (0.964) | 0.050 (0.896) | 0.052 (0.757) | 0.072 (0.939) |
| CC1/2  | 0.999 (0.369) | 0.998 (0.441) | 0.999 (0.504) | 0.999 (0.531) | 0.997 (0.389) |
| ⟨I/σI⟩  | 14.6 (1.0) | 12.3 (1.0) | 10.9 (1.0) | 12.6 (1.0) | 10.1 (1.0) |
| No. of reflections |                       |                       |                      |                      |                  |
| Observed       | 1,009,308             | 336,646               | 351,626              | 529,506              | 750,538          |
| Unique         | 71,625 (3952)         | 42,052 (4328)         | 51,984 (4215)        | 38,760 (4666)        | 51,324 (4116)    |
| Completeness (%) | 98.8 (86.8)         | 100.0 (100.0)         | 99.9 (99.9)          | 99.9 (100.0)         | 99.1 (98.7)      |
|Multiplicity    | 14.1 (11.4)           | 8.0 (8.2)             | 6.8 (7.0)            | 13.7 (13.1)          | 14.6 (14.9)      |
| Refinement     |                       |                       |                      |                      |                  |
| Resolution (Å)  | 41.81–1.90            | 48.83–2.63            | 28.65–2.12           | 29.43–2.70           |                  |
| No. of reflections | 67,965               | 39,931                | 49,340               | 36,717               |                  |
| R cryst/R free | 0.212/0.243           | 0.231/0.288           | 0.238/0.274          | 0.227/0.276          |                  |
| No. of αβ-heterodimer in ASU | 2                  | 4                    | 2                    | 4                    |                  |
| Protein residues | 571                | 1130                 | 568                  | 1131                 |                  |
| No. of atoms   | 4717                 | 8453                 | 4584                 | 8680                 |                  |
| Protein        | 4450                 | 8439                 | 4434                 | 8655                 |                  |
| Detergent/ion  | 88                   | –                    | 60                   | 15                   |                  |
| Water          | 179                  | 14                   | 90                   | 10                   |                  |
| B-factors (Å²) | 42.89                | 71.24                | 51.19                | 74.18                |                  |
| Protein        | 42.45                | 71.28                | 51.14                | 74.17                |                  |
| Detergent/ion  | 71.49                | –                   | 68.09                | 98.59                |                  |
| Water          | 42.14                | 51.36                | 42.27                | 52.06                |                  |
| R.m.s deviations |                    |                      |                      |                      |                  |
| Bond lengths (Å) | 0.010              | 0.007                | 0.008                | 0.007                |                  |
| Bond angles (°) | 1.579               | 1.430                | 1.538                | 1.514                |                  |
| Ramachandran   |                       |                       |                      |                      |                  |
| Favored (%)    | 96.3                 | 95.1                 | 96.4                 | 93.9                 |                  |
| Allowed (%)    | 3.7                  | 5.7                  | 3.6                  | 5.8                  |                  |
| Outliers (%)   | 0                    | 0.4                  | 0                    | 0.4                  |                  |
ΔH2 and ΔH3, and the level of mature PSD was low overall. Notably, uncleaved ΔH3 was enriched in the soluble fraction, and the cleaved form was not detected at a significant level in all fractions. The concentration of hydrophobic residues on the surface of the N-terminal helices further supports that the N-terminal domain is a hot spot for membrane association (Fig. 3C and Supplementary Fig. S5). Interestingly, DDM molecules identified in the structure of Apo-PSD1 interact with the positively charged surface of PSD through the relatively polar disaccharide moiety, whereas the dodecyl tail of the detergent interacts with the hydrophobic surface of the enzyme encompassing the N-terminal helices (Supplementary Fig. S6). These molecular interactions may mimic those occurring on the cellular membranes, where PSD is partially embedded within a single layer of phospholipids using the N-terminal helical domain.

**Phospholipid-bound structures.** To identify the molecular determinants required for substrate recognition and binding, we first attempted to form a stable covalent linkage between PSD and a phospholipid via sodium cyanoborohydride (NaCNBH₃)-dependent reduction of a Schiff base intermediate (Fig. 4A)¹⁹. Conjugation was tested using 8:0/8:0 PE (8PE), 10:0/10:0 PS (10PS), and 14:0/14:0 PS (14PS) with full-length and truncated PSD, and the results were analyzed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Incubation of PSD with a phospholipid increased the mass-to-charge ratio (m/z) of the α-subunit by 450.5, 506.7, and 618.8 for 8PE, 10PE, and 14PE, respectively, which is consistent with the formation of conjugates with the corresponding PE (Fig. 4B). The results indicate that the reduction of the Schiff base occurred after decarboxylation. MS data confirmed the successful modification of the protein with an efficiency greater than 94%: 98%, 97%, and 94% conjugation for 8PE, 10PE, and 14PE, respectively. Among these samples, 8PE- and 10PE-linked PSD₁₋₂₈₇ yielded crystals, which diffracted to 2.12 Å and 2.70 Å resolution, respectively. These X-ray diffraction data were used to determine the structures shown in Fig. 4C; they...
are further denoted as 8PE-PSD and 10PE-PSD, respectively. The asymmetric unit of 8PE-PSD contains two αβ-heterodimers, whereas that of 10PE-PSD contains four.

The overall conformation of PSD in both PE-bound structures is highly homologous to that in apo structures (r.m.s.d. of Cαs = 0.218 Å for 8PE-PSD; 0.399 Å and 0.390 Å for the 10PE-PSD αβ-heterotetramer). Similar to Apo-PSD1, one of the two DDM molecules was modeled at the interface between the two β-subunits in 8PE-PSD. Charge distribution on the enzyme surface demonstrates that the substrate-binding site creates a predominantly hydrophobic environment optimal for accommodating fatty acyl chains of glycerophospholipids (Fig. 4D). Solid electron densities were observed in all subunits of PE connected to Pvl-254. However, the quality of local electron densities around fatty acyl chains varies from one subunit to another. For example, in one αβ-heterodimer of 8PE-PSD, 12 out of 16 carbon atoms could be modeled in the fatty acyl group of 8PE, whereas only 9 were traceable in the other. Although we were able to observe the electron densities of two fatty acyl chains connected to the glycerol backbone, the sn-1 and sn-2 positions on the glycerol backbone could not be determined unambiguously with the present data even at a resolution of 2.12 Å. The assignment was based on the electron density in chain B of the 8PE-PSD structure, which was considered the best. The sn-2 acyl chains were modeled in close proximity to the hydrophobic protein surface, which was composed of Val-37, Phe-41, Phe-63, Phe-67, and Leu-252, with sn-1 acyl chains extending near the surface defined by Leu-13, Leu-18, and Thr-204. In general, the fatty acyl chain at the sn-2 position is more ordered than that connected to sn-1 in the present structure. For instance, the entire sn-2 acyl chains could be modeled in 10PE-PSD, whereas most sn-1 acyl chains were disordered because of the relatively shallow binding pocket. In both structures, the sn-2 acyl chains extend to the hydrophobic surface of the N-terminal helical domain, where the membrane association is presumed to occur, as described earlier. The sn-2 chain-binding site, which is composed of hydrophobic residues, allows for non-specific binding
with a fatty acyl chain. This relaxed specificity is expected to be effective for the sn-1 chain binding site as well, in which the docked fatty acyl chain exhibits a greater degree of conformational flexibility. The binding mode of fatty acyl chains observed in our structures reflects the intrinsic nature of PSD-lipid interactions and are not crystallization artefacts, since there are no neighboring symmetrically related molecules, which would have affected the protein-lipid interactions.

Recognition of phosphoserine moiety. The phosphate group of PE forms hydrogen bonds with the side chain of Tyr-137 and the backbone amide group of Val-167 in both PE-bound structures, and an additional hydrogen bond is identified with the side chain of Ser-166 in 10PE-PSD (Fig. 5A). The orientation of Pvl-254-linked PE in both structures suggests that His-144 is likely to interact with the departing carboxyl group of PS. To examine whether these amino acid residues are critical for decarboxylation activity, we performed in vitro assays using PSD1–322 variants containing a site-specific mutation, followed by LC–MS analysis (Fig. 5B). When Ser-166 was substituted with alanine, however, no significant change from the wild-type protein was observed in the in vitro assay. Meanwhile, Y137F was able to decarboxylate PS at a reduced rate (~50%). Tyr-137 is highly conserved among the PSD family (85%) or occasionally replaced by arginine (15%). Interestingly, when both Tyr-137 and Ser-166 were mutated, the double mutant protein Y137F/S166A exhibited a substantially lower activity compared to the wild-type protein, demonstrating the combinatorial effects of these residues in interacting with the phosphate moiety of the lipid substrate. Mutation of the absolutely conserved His-144 completely

![Figure 4. Phospholipid-bound structures. (A) A scheme of NaCNBH₃-dependent reduction of Schiff base. Ptd, phosphatidyl moiety (B) MALDI-ToF analysis of phospholipid conjugation on α-chain. The MS peaks of apo and modified α-chains are marked as gray dashed lines and black arrows for comparison of the m/z values. Observed and calculated m/z values of conjugates are 4925.6, 4926.6 (8:0/8:0 PE-conjugated), 4981.8, 4980.7 (10:0/10:0 PE-conjugated), and 5093.8, 5092.8 (14:0/14:0 PE-conjugated), respectively. (C) A zoomed-in view of linked phospholipid from 8PE-PSD (Left) and 10PE-PSD (Right). A Fo–Fc omit map of the phospholipid is illustrated as mesh contoured at 2.5 σ. Carbon atoms of the lipid are displayed in yellow, oxygen in red, nitrogen in blue, and phosphorous in purple. (D) Overlay of the acyl-chains of bound lipid in the hydrophobic pocket from all four subunits of 10PE-PSD structure. Hydrophobic residues surrounding the acyl-chains are labeled and highlighted as light blue stick.](https://www.nature.com/scientificreports/
abolished the decarboxylation activity; neither H144N nor H144A could support the formation of PE in the assay. Considering the importance of His-144, we investigated the specific role of this residue in catalysis by testing whether His-144 mutants were able to form a conjugate product with PS and PE (Fig. 5C). Although the H144A and H144N mutants did not conjugate with 10PS or 14PS, these proteins were able to form an adduct with 8PE, albeit at a reduced efficiency compared to the wild-type protein. Collectively, the data suggest that His-144 mutants were not able to form a stable Schiff base with PS, highlighting the critical role of this residue in correctly recognizing the carboxyl group of substrate lipids.

Mutagenesis studies on the mechanism of auto-cleavage. Because PSD is a member of the serine protease family and is known to require a catalytic triad for auto-cleavage\(^{22,23}\), we investigated the amino acid residues that are responsible for the maturation of the proenzyme on the basis of genetic and structural informa-
Ser-308, His-198, and Asp-139 have been reported to organize the catalytic triad of PSD in *P. knowlesi* (corresponding to Ser-254, His-147, and Asp-90 in *E. coli*, respectively); equivalent residues in *S. cerevisiae* are Ser-463, His-345, and Asp-210 (Ser-254, His-144, and Asp-90 in *E. coli*, respectively) (Supplementary Fig. S7)\(^2^2\),\(^2^3\). Candidate residues for the maturation of *E. coli* PSD were selected for mutation, and the auto-cleavage of the mutant proteins was analyzed from cell lysates after a 4 h induction period (Fig. 6A). Additionally, we purified the recombinant proteins from cell cultures incubated overnight for induction and analyzed the result of the cleavage, which occurred approximately 24 h after the induction of protein expression had begun. Alanine mutation of Ser-254 completely abolished auto-cleavage, which is consistent with a previous report\(^2^5\). Similarly, a single mutation of His-144 to alanine or asparagine appeared initially to hamper the auto-proteolysis of the proenzyme; however, both mutants were identified as substantially cleaved in the purified form. An unknown cleavage product with a size slightly smaller than that of the proenzyme was detected from the lysates for both H144A and H144N. However, this unknown fragment was insoluble and not observed after purification. For the H147N and H147A mutants, a significant cleavage occurred after 4 h of induction, which became nearly complete after the purification steps. Similarly, in single mutant proteins of Asp-90, most of the proenzyme was cleaved initially, and the completely processed PSD was identified after purification. However, purified D90N or D90A mutant proteins did not compromise PS decarboxylation activity, unlike His-144 mutants (Fig. 6B). Since the side chains of Asp-90 and His-144 are ~ 9 Å apart in the crystal structures, Asp-90 may not be optimally positioned to depolarize His-144. In the vicinity of the histidine residue, Asp-142 is located at ~ 6 Å; Asp-142 has not been considered as a component of the catalytic triad of PSD in the past. We tested this residue for auto-cleavage by mutating it to asparagine or alanine; however, the results were nearly identical for these mutants and Asp-90 mutants. The results of these single mutants in the pro-enzyme processing suggest that His-144 and His-147 may act as complementary components of the D-H-S triad for *E. coli* PSD, as may Asp-90 and Asp-142. To investigate this possibility, we constructed and analyzed double mutant proteins of these pairs, that is, H144A/H147A and D90A/D142A. Surprisingly, the D90A/D142A double mutant did not undergo auto-cleavage and remained as a proenzyme in the lysates, although became cleaved in the purified form. Meanwhile, the result from H144A/ H147A mutant was quite similar to that of H144A; the double mutant was not able to auto-cleave initially, yet displayed significant cleavage products after purification steps. Therefore, our single- and double-mutation experiments support the hypothesis that His-144 plays a major role in activating Ser-254, whereas Asp-90 and Asp-142 can function in complementary to each other in the proenzyme maturation process.

Lastly, we introduced mutations at Gly-253 and Thr-255, which are highly conserved amino acids located next to the nucleophilic Ser-254. Together, these residues comprise the LGST motif, the consensus sequence conserved among the PSD family on which auto-cleavage occurs. G253A, G253P, and T255A could not form auto-cleaved

**Figure 6.** Key residues required for auto-cleavage of proenzyme. (A) Putative active site for auto-cleavage is shown where amino acid residues selected for mutagenesis are highlighted as sticks (Left). Wild-type and site-specific mutant proteins are visualized by immunoblot from cell lysates after 4 h induction (Top Right), and the purified recombinant proteins are analyzed by Coomasie staining on 12% SDS-PAGE gel (Bottom Right). Proenzyme (theoretical molecular weight, 36.9 kDa), processed β-chain (28.6 kDa), and α-chain (8.3 kDa). Lane M ladder marker. (B) LC–MS analysis of PS decarboxylation activity of Asp-90 mutants. Mean and standard deviation are plotted. Experiments were performed in triplicates.
products after 4 h of induction; however, G253A and T255A displayed approximately 70% cleavage after a longer period, as identified in the purified form. G253P remained uncleaved in its purified form, similar to S254A.

**Discussion**

Previous studies have shown that substrate analogs lacking fatty acyl chains, such as serine, phosphoserine, or glycerol phosphoserine were not decarboxylated by PSD, underscoring the importance of essential hydrophobic interactions with fatty acyl chains for substrate binding and catalysis. Our high-resolution structures of PE-bound PSD reveal detailed molecular interactions between the protein and the phospholipid substrate: one fatty acyl chain binds to the larger hydrophobic protein surface defined by Val-37, Phe-41, Phe-63, Phe-67, and Leu-252, whereas the other acyl chain is found on the relatively smaller surface composed of Leu-13, Leu-18, and Thr-204. It is not feasible to unambiguously discriminate the $\text{sn}_{1}$- or $\text{sn}_{2}$-isomers of the bound phospholipid, and our structural data suggest that both binding modes may be plausible; that is, the $\text{sn}_{1}$- or $\text{sn}_{2}$- acyl chain is not restricted to binding at a particular site of the enzyme. Although fatty acyl chains are an essential component for effective binding to PSD, it appears that the enzyme mainly recognizes one of the fatty acyl chains via non-specific hydrophobic interactions, whether it is an $\text{sn}_{1}$- or $\text{sn}_{2}$- acyl chain. Phospholipids are highly diverse in length and degree of saturation of fatty acyl chains, where 258 different species have been experimentally identified in *E. coli* to date. As the sole enzyme for synthesizing PE in bacteria, PSD must act promiscuously on a wide range of PS. Current structural data provide insights into the broad specificity of PSD, where the lipid-binding surface permits non-specific hydrophobic interactions with various types of fatty acyl chains of the phospholipid. Additional molecular determinants for PS binding are Tyr-137, His-144, and Ser-166, which interact with phosphate and carboxyl groups. Our structural and biochemical studies clearly support the formation of Schiff base intermediates on the reaction coordinate and suggest that His-144 is likely to interact with the departing carboxyl group of PS, which was also shown to be critical in the formation of a stable Schiff base intermediate (Fig. 7A).

Another valuable feature highlighted in the present structures is that PSD is a monotopic membrane protein that is embedded into a single face of the membrane. Structures of monotopic membrane proteins are extremely rare, accounting for only ~0.06% of the total non-redundant structures. We propose that heterotetrameric PSD employs coplanar amphipathic helices in the N-terminus of beta-subunits to associate with the cell membrane, as illustrated in Fig. 7B. Estimation of the protein-membrane border in bacterial cells is further aided by co-crystallized DDM, the length of which roughly encompasses a single membrane leaflet (Supplementary Fig. S8). In this model, the enzyme is in proximity to the membrane interface, similar to phosphoglycosyl transferase, PglC, another example of monotopic membrane protein.

During the preparation of this manuscript, Watanabe et al. reported two crystal structures of *E. coli* PSD in apo and PE-bound states at resolutions of 2.6 and 3.6 Å, respectively. The conclusions drawn by the authors are largely consistent with ours, such as the biological assembly of the enzyme, membrane association of the enzyme, and biochemical properties of key residues contributing to phospholipid recognition. Our high-resolution
structures provide more detailed insights into the protein-lipid interactions, indicating the precise active sites on the enzyme where the acyl chains of phospholipids bind. Furthermore, our in-depth analyses of the auto-cleavage of proenzymes required for maturation identified critical residues comprising a D-H-S catalytic triad for *E. coli* PSD: His-144 appears to be the major player in enhancing the nucleophilicity of Ser-254, whereas Asp-90 and Asp-142 can functionally complement to each other during the maturation process. Despite H144A, H144N, H144A/H147, and D90A/D142A were not able to auto-cleave initially, these mutant proteins were eventually cleaved to a certain degree after a longer period, suggesting an alternative mechanism may exist for the activation of the proenzyme. In summary, our data support that the auto-cleavage of the *E. coli* PSD is mainly achieved by D90/D142–H144–S254, analogous to a conventional D-H-S catalytic triad.

The LGST motif, a signature cleavage site conserved among bacterial PSD, is predicted to form a short loop connecting two beta sheets in the proenzyme. It has been proposed that the relief of conformational strain across the cleavage site in proenzymes may be a driving force for auto-cleavage in other pyruvoyl-dependent decarboxylases, including *S. adenosylmethionine decarboxylase*33, aspartate decarboxylase11,12, and histidine decarboxylase33. Our mutagenesis studies showed that G253A, G253P, and T255A exhibited significantly reduced auto-cleavage, suggesting the importance of the correct conformation of the loop and positioning of the serine hydroxyl group for proenzyme maturation. Because of the apparent structural reorganization of the processed α- and β-subunits in the current structures, it is challenging to precisely locate and orient the key residues in the 3-D space prior to the auto-cleavage event. The determination of the pro-PSD structure will provide critical insights regarding the activation process.

The present structural information will be highly valuable in developing a novel class of antibiotics because numerous pathogens are known to require PSD for viability. Additionally, the activity of a mammalian homolog has been reported to be important. In an animal model, mouse embryos lacking *psd*1 did not survive past 9 days of development35. Human PISD has been associated with numerous diseases including various cancers36–38, Parkinson’s disease39, Alzheimer’s disease40, liver disease41, candidiasis42–45, and malaria46,47. The physiological significance of PISD has been demonstrated in tumor-initiating cells, where overexpression of PISD downregulates mitochondrial function and inhibits tumor growth37. Notably, recent studies demonstrated an emerging role of PISD in tumor regulation, where the tumor repressor LACtB downregulates PISD levels, leading to the alteration of mitochondrial lipid metabolism and differentiation of certain cancer cells35. Therefore, the use of therapeutic compounds targeting human PISD may serve as an effective strategy for treating related diseases, including cancer. The structural and functional information from *E. coli* PSD can be extended to understand and predict the biological behaviors of mammalian homologs.

**Methods**

**Materials.** For membrane protein solubilization, detergent dodecyl b-D-maltopranoside (DMM) was purchased from Carbosynth, UK (cat. no. DD06199). For conjugation or co-crystallization of phospholipids, 8/8:0 PE (8PE; cat. no. 850699), 8/0:8 PS (8PS; cat. no. 840031), 10/0:10 PS (10PS; cat. no. 840036), 14/0:14 PS (14PS; cat. no. 840033), and 16/0:18.1 PS (cat. no. 840034) was purchased from Avanti Polar Lipids, Alabama. Soy PS was purchased from Sigma-Aldrich, Missouri (cat. no. P0474) for functional assay. 2,5-Dihydroxybenzoic acid (2,5-DHB) (part no. 8201346) was purchased from Bruker, Massachusetts for Matrix-Assisted Laser Desorption Ionization (MALDI) analysis.

**Cloning.** Plasmid encoding wild type *E. coli* psd gene was purchased from NBRP, Shizuoka, Japan (Resource No. JW4121-AM)48. To generate full-length PSD expressing vector, pLATE31-PSD1–322, the gene was amplified by the following primers: PSD_ECOLI_1–322_pLATE31 and PSD_ECOLI_1–322_pLATE31 and cloned into the pLATE31 vector. The sequences were verified by DNA sequencing at the Macrogen (Seoul, South Korea). All site-directed point mutations were introduced to full-length psd1 DNA sequencing at the Macrogen (Seoul, South Korea). For expression of the PSD1–287, E. coli BL21(DE3) transformed by the pLATE31-PSD1–287 vector was incubated in Luria–Bertani (LB) medium supplemented with 100 μg/mL of ampicillin at 37°C until OD600 = 0.3–0.5. Expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM, and the cells were incubated at 20°C for 19–20 h with shaking at 160 rpm. The cells were harvested by centrifugation at 11,355 rcf (8000 rpm), 4°C for 10 min, resuspended by buffer A (30 mM Tris–HCl pH 7.5, 150 mM NaCl, and 10% v/v glycerol), and pelleted at 3214 rcf, 4°C for 30 min. The cell pellets were stored at −86°C until used. The cell pellets were lysed by sonication in buffer B (50 mM Tris–HCl pH 7.5, 500 mM NaCl, 10% (v/v) glycerol, and 50 mM imidazole supplemented with 2 mM dithiothreitol (DTT), LPS Solution; Daejeon, South Korea), 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich) and 1 mg/mL lysozyme (Sigma-Aldrich)]. To solubilize membrane protein, the lysates were incubated with DDM to a final concentration of 1% (w/v) at 4°C for 1 h with shaking. The solubilized lysates were centrifuged 18,686 rcf (13,000 rpm), 4°C for 30 min, filtered through 0.2 μm pore size syringe filter, and loaded onto HisTrap HP 5 mL column (GE Healthcare, Illinois) equilibrated with buffer C (50 mM Tris–HCl pH 7.5, 500 mM NaCl, 10% (v/v) glycerol, 50 mM imidazole, and 0.1% (w/v) DDM). The PSD was eluted with 500 mM imidazole gradient in buffer C. The fractions were further purified by size-exclusion chromatography (SEC) on a HiLoad 16/600 Superdex 75 pg column (GE Healthcare) equilibrated with buffer A supplemented with 0.05% (w/v) DDM. The
Ligand conjugation by reduction for structural characterization. Schiff base reduction was performed as described by Li and Dowhan with minor changes. Prior to the reduction, purified PSD1–287 was buffer exchanged by SEC on a HiLoad 16/600 Superdex 75 pg column to buffer D (30 mM HEPES pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, and 0.05% (w/v) DDM). To conjugate the ligands to the protein, 2.4–2.5 mg of 38.7 μM PSD1–287, 5 μM NaCNBH3, and 10 mM 8PE/10PS/14PS were sequentially mixed to a final concentration of 8.8 mg/mL of 10PS-conjugated PSD1–287 in 0.2 M lithium sulfate monohydrate, 0.1 M Tris pH 7.0. However, there was no measurable electron density of the lipid. 8PE-bound PSD1–287 crys-277 tals were formed at a concentration of 8.0 mg/mL of 8PE-conjugated PSD1–287 prepared above in 0.1 M potassium thiocyanate and 30% (w/v) PEGMME 2000. 10:0/10:0 PE(10PE)-bound PSD1–287 crystals were observed at a concentration of 8.8 mg/mL of 10PS-conjugated PSD1–287 in 0.2 M lithium sulfate monohydrate, 0.1 M Tris pH 8.5, 25% (w/v) PEG 3350. SeMet substituted Apo-PSD1 crystals were formed with rod-shaped at a concentration of 9.7 mg/mL in the identical condition to that of the unlabeled sample.

All crystals were cryoprotected by mother liquor supplemented with 20% (v/v) glycerol and flash-frozen in liquid nitrogen. X-ray diffraction data were collected under cryogenic conditions using the ADSC Q270 detector at Pohang Accelerator Laboratory (PAL; Pohang, South Korea) beamline 7A (SeMet, Apo-PSD1 and 2) or using Eiger X 9 M detector at PAL beamline 5C (8PE- and 10PE-bound PSD1–287) (Table S1). SeMet data were indexed, integrated, scaled by HKL2000. All native data were processed by XDS and merged by Aimless in CCP4 suite. To solve the phase problem, the processed SeMet data were used for Single-wavelength Anomalous Dispersion (SAD) phasing by AutoSol in Phenix suite. Apo-PSD1 structure was built by iterative runs of real space equilibrating against 60 μL of the reservoir solution. Rod-shaped Apo-PSD1 crystallized at a concentration of 38.7 μM PSD1–287, 5 μM NaCNBH3, and 10 mM 8PE/10PS/14PS were sequentially mixed to a final concentration of 22.5 μM. The mixture was incubated in a water bath at 37 °C for 10 h (PSD1–322) or 14 h (PSD1–287), and buffer exchanged by a 3 K MWCO 15 mL Amicon Ultra centrifugal filter to the buffer A supplemented with 0.05% (w/v) DDM. The conjugation efficiency was estimated by MALDI analysis.

Membrane association assay. E. coli BL21(DE3) cells containing wild-type PSD1–322 (WT) or truncation variants (ΔH1, 13–322; ΔH2, 30–322; ΔH3, 46–322) expressing plasmids were incubated in 1 L of LB medium supplemented with 100 μg/mL of ampicillin. When OD600 reaches ~0.1, the protein expressions were induced by addition of IPTG to a final concentration of 0.1 mM. The flask containing the expression cell was incubated either at 20 °C (WT, ΔH2, and ΔH3) or 37 °C (ΔH1) until OD600 reaches 1.0–1.5 shaking at 160 rpm. The cells were harvested and stored as described above. The cell pellets were lysed in 1 × PBS (3 mM Na2HPO4, 1.1 mM KH2PO4, and 160 mM NaCl, pH 7.4; LPS Solution, Republic of Korea) and sonicated. Inclusion body was separated by centrifugation at 21,672 rcf (14,000 rpm) and lysed in 25 mL of 1 × PBS. The supernatant was applied to ultracentrifuge at 120,000 rcf (34,200 rpm, Ti 70 rotor equipped with Optima L-100K, Beckman Coulter, USA)
for 1 h. After the ultracentrifuge, the supernatant (soluble fraction) and the pellet (membrane fraction) were collected. The pellet was lysed in 25 mL of 1× PBS. Each fraction was analyzed by 12% SDS-PAGE and visualized by western blotting using HRP Anti-6 × His tag antibody (cat. no. ab1187, Abcam, UK). Original images of the membrane containing blots are shown in Supplementary Fig. S9.

**LC–MS analysis of functional activity.** The enzymatic reaction was initiated by mixing PSD$_{1–287}$ and soy PS in buffer A supplemented with 0.05% (v/v) DDM to a final concentration of 10 μM and 1 mM, respectively, and incubated in a water bath at 37 °C for 30 min. The reaction mixtures were quenched by adding an equal volume of 1 M HCl. Subsequently, lipids in the reaction samples were extracted by Folch’s method$^{32}$. Briefly, one volume of the quenched mixtures was transferred to 5 volumes of the chloroform/methanol (2:1, v/v) in a glass tube. The tube vortexed and centrifuged briefly at 500 rcf, 4 °C for 10 min. By carefully discarding the upper phase, the lower phase was collected in a new glass tube and dried out. The extracted lipids were dissolved in methanol and subjected to LC–MS analysis. An aliquot of 20 μL of the sample was injected into a reversed phase column (ZORBAX RR Eclipse Plus C18, 95 Å, 4.6 × 100 mm, 3.5 μm; Agilent Technologies) attached to Agilent 1260 Infinity Quaternary LC system (Agilent Technologies). Chromatographic separation was performed by using Solvent A (10 mM ammonium acetate in acetonitrile:water 60:40 (v/v)), and solvent B [10 mM ammonium acetate in isopropanol:acetonitrile 90:10 (v/v)], using a gradient step with the flow rate of 0.8 ml/min as following: (1) 70% B for 1 min (column equilibration); (2) a linear gradient from 70 to 100% B for 11 min; (3) a hold at 100% B for 1 min (column wash); (4) a linear gradient from 100 to 70% B for 1 min; (5) a hold at 70% B for 1 min (column equilibration) with additional hold for 5 min using Post time. Eluted fractions were loaded to the MS and MS/MS system (Agilent 6520 Q-TOF LC/MS; Agilent Technologies) and analyzed in the positive ionization mode utilizing Dual ESI as an ionization source. Major phospholipids were identified and relatively quantified by LipidBLAST$^3$. Results were visualized by GraphPad Prism$^{35}$. Functional assay of the PSD$_{1–322}$ was performed in the same manner as the PSD$_{1–287}$ with final enzyme concentrations for the reaction reduced to 1 μM. For conversion rate analysis of wild type and mutants PSD$_{1–322}$, 1 μM of the enzyme is incubated with 1 mM 16:0/18:1 PS in a 37 °C water bath for 0, 5, 10, 15, and 20 min. Following extraction of phospholipids and LC–MS analysis were performed as described above.

**Ligand conjugation by reduction of His-144 mutants.** Conjugation assay was performed similarly as used for the structure characterization described above with minor changes. Buffer exchanged PSD$_{1–322}$ variants (H144A and H144N) were concentrated to 0.431 mg/mL (H144A) or 0.901 mg/mL (H144N). The protein, 5 mM NaCNBH$_3$, and 10 mM 8PE/10PS/14PS were sequentially mixed with a volume ratio of 89:1:10 while vortexing. The mixture was incubated in a water bath at 37 °C for 15 h and analyzed by MALDI-ToF.

**MALDI-ToF for conjugation efficiency analysis.** For preparation of matrix solution, 2,5-DHB was dissolved in acetonitrile/0.1% (v/v) TFA (30:70, v/v; TA30) to a final concentration of 20 mg/mL. Approximately 1 mg/mL purified protein samples were tenfold diluted by adding 0.1% (v/v) trifluoroacetic acid (TFA). The diluted solution was mixed with an equal volume of the matrix solution. Then, a 0.5 μL of the mixture was spotted to MTP 384 ground steel BC targets. The target plate was loaded to autoflex speed TOF/TOF (Bruker). Measurements were conducted on linear positive ion mode using flexControl program at laser frequency of 2000 Hz. For each dataset, 2000 shots were collected by random walking and merged.

**Auto-activation assay.** _E. coli_ cells expressing wild-type or site-specific variants were incubated in a 20 mL LB media with 100 μg/mL ampicillin and induced when OD$_{600}$ is 0.4–0.6 and incubated for 4 h at 20 °C. The cells were harvested by centrifugation at 4 °C, 3214 rcf (3900 rpm). The pellets were lysed in 700 μL of 1× PBS and analyzed by 12% SDS-PAGE and western blotting with the anti-histag antibody. For the assay of the purified proteins, the expression of the protein was induced at 20 °C for 19–20 h. After the cell harvest, the protein undergoes purification process at room temperature which usually took ~ 24 h. The purified proteins were analyzed by 12% SDS-PAGE and stained by Coomassie blue.

**Data availability**

The atomic coordinates and structure factors for Apo-PSD1, Apo-PSD2, 8PE-PSD, and 10PE-PSD are deposited to Protein Data Bank under accession code 7CNW, 7CNX, 7CNY, and 7CNZ, respectively.

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Author contributions

J.K. and G.C. contributed to project design and conception, G.C. performed cloning, expression, purification, conjugation of ligands, crystallization, data collection, model building, LC–MS and MALDI–ToF analysis. E.L. performed sample preparation for SEC–MALS analysis and assisted membrane association and auto-activation assay. G.C. and J.K. analyzed data and wrote the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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