An improved and highly selective fluorescence assay for measuring phosphatidylserine decarboxylase activity

Received for publication, March 11, 2020, and in revised form, May 14, 2020. Published, Papers in Press, May 19, 2020, DOI 10.1074/jbc.RA120.013421

Jae-Yeon Choi¹, Raymond Black III¹, HeeJung Lee¹, James Di Giovanni², Robert C. Murphy², Choukri Ben Mamoun³, and Dennis R. Voelker¹*.

From the ¹Basic Science Section, Department of Medicine, National Jewish Health, Denver, Colorado, USA, the ²Department of Pharmacology, University of Colorado Denver, Aurora, Colorado, USA, and the ³Section of Infectious Diseases, Department of Internal Medicine, Yale School of Medicine, New Haven, Connecticut, USA.

Edited by Karen G. Fleming

Phosphatidylserine decarboxylases (PSDs) catalyze the conversion of phosphatidylserine (PS) to phosphatidylethanolamine (PE), a critical step in membrane biogenesis and a potential target for development of antimicrobial and anti-cancer drugs. PSD activity has typically been quantified using radioactive substrates and products. Recently, we described a fluorescence-based assay that measures the PSD reaction using distyrylbenzene-bis-aldehyde (DSB-3), whose reaction with PE produces a fluorescence signal. However, DSB-3 is not widely available and also reacts with PSD’s substrate, PS, producing an adduct with lower fluorescence yield than that of PE. Here, we report a new fluorescence-based assay that is specific for PSD and in which the presence of PS causes only negligible background. This new assay uses 1,2-diacetyl benzene/β-mercaptoethanol, which forms a fluorescent iso-indole-mercaptide conjugate with PE. PE detection with this method is very sensitive and comparable with detection by radiochemical methods. Model reactions examining adduct formation with ethanolamine produced stable products of exact masses (m/z) of 342.119 and 264.105. The assay is robust, with a signal/background ratio of 24, and can readily detect formation of 100 pmol of PE produced from Escherichia coli membranes, Candida albicans mitochondria, or HeLa cell mitochondria. PSD activity can easily be quantified by sequential reagent additions in 96- or 384-well plates, making it readily adaptable to high-throughput screening for PSD inhibitors. This new assay now enables straightforward large-scale screening for PSD inhibitors against pathogenic fungi, antibiotic-resistant bacteria, and neoplastic mammalian cells.

PE (phosphatidylethanolamine) is an essential membrane phospholipid for many Gram-negative prokaryotes and eukaryotes (1–3). In Escherichia coli, PE is the most abundant phospholipid present in the cytoplasmic membrane and the inner leaflet of the outer membrane (4). In yeast, PE is the second most abundant phospholipid constituent of cell membranes and also serves as an important precursor for phosphatidylcholine synthesis, via SAM-dependent methylation of its primary amine (5). In mammalian cells, PE is typically the second most abundant phospholipid found in nearly all cell membranes (6). Multiple synthetic pathways can give rise to PE pools (7) in yeast and mammalian cells. In E. coli, PSD activity is an exclusive source of PE, whereas in yeast, PE can be produced via two spatially segregated PSDs (8, 9), the CDP-ethanolamine-dependent “Kennedy pathway” (10), or the lysophospholipid acylation pathway (11). In mammalian systems, the Kennedy pathway, lysophospholipid-acylation pathway, PSD pathway, and base-exchange pathways can all contribute to the total cellular pool of PE (12).

Despite redundancies in synthetic pathways resulting in PE formation in eukaryotes, not all pathways can satisfy specific needs of all organelles for their required complement of PE. For example, the PE content of mitochondria is essential for the function and maintenance of the organelle, and deletion of the nuclear gene encoding mitochondrial PSD results in ethanolamine auxotrophy, respiratory deficiency, and mitochondrial instability in yeast (8, 9) and embryonic lethality in mice (13). Even modest decline of mammalian mitochondrial PE content (<30%) resulting from RNAi silencing of PSD in Chinese hamster ovary cells was shown to affect mitochondrial structure and function and to impair cell viability (14). More recently, a missense variant c.797G→A/p.(Cys266Tyr) in PISD (the mammalian genomic designation for PSD) was found in some patients with spondyloepimetaphyseal dysplasias, a rare form of dwarfism, characterized by abnormal vertebral bodies and epimetafysial abnormalities (15). Patient-derived fibroblasts showed fragmented mitochondrial morphology and altered mitochondrial function.

Additional recent studies have shown that PSD plays a key role in cancer development (16, 17). Keckesova et al. (16) have shown that the mitochondrial protein, a serine β-lactamase–like protein (LACTB), acts as a tumor suppressor that inhibits the proliferation of certain breast cancer cells, and the suppression works through the inhibition of mitochondrial lipid synthesis. When LACTB was overexpressed in the tumor cells, there was a 30–50% reduction in lyso-PE and PE content. Supplementation with lyso-PE after LACTB overexpression, however, bypassed the inhibition of cell proliferation by LACTB. Once inside the cell, lyso-PE is readily acylated to form PE (11, 18). The reduction of the lipids by LACTB overexpression was due to decreased amounts of PSD protein (by 60–95%) in mitochondria (16, 18). In contrast, Chen et al. (17) have reported that the mammalian PISD gene was down-regulated by a factor of 8 in mouse breast cancer stem cell lines and provided evidence of PISD as a novel regulator of tumor-initiating cells.
Fluorescence detection of PS decarboxylase activity

When PISD was overexpressed, the tumor-initiating potential of cancer cells was greatly reduced.

Despite emerging evidence for the critical role PSD plays in membrane biogenesis in both prokaryotes and eukaryotes, no selective inhibitors for this family of enzymes have been described. This absence of PSD inhibitors is likely a consequence of the relatively cumbersome nature of current assays available for screening for inhibitors of the enzyme. We have recently begun developing new assays for the enzymes of phospholipid and sphingolipid synthesis, amenable to high-throughput screening (HTS) for the purpose of discovering inhibitors that may prove useful as antimicrobial and antineoplastic agents. To pursue this goal, we have engaged in developing new assays for phospholipid and sphingolipid synthetic enzymes, which are compatible with HTS. In a recent report, we described the first fluorescence assay for PSD catalysis, which can be used for HTS, using the bis-aldehyde reagent DSB-3 (19). Recent application of this assay led to the discovery of five inhibitors of the PSD enzyme (20).

The purpose of undertaking the work in this paper was to 1) identify new and more widely available reagents and methods for detection of PSD catalysis that would improve upon some of the shortcomings of the DSB-3 method, 2) characterize the properties and selectivity of the new method, and 3) test the feasibility of application of the new method to crude preparations of the enzymes in bacteria, fungi, and neoplastic cells.

The DSB-3 compound is not commercially available, and synthesis in a molecular biology laboratory can be quite challenging. The cost of DSB-3 synthesis through a chemical company can be quite high (~$10,000). Autofluorescence of DSB-3 increases in the presence of the detergent Triton X-100, which is required for PSD catalysis. DSB-3 also forms fluorescent adducts with PS, the substrate for PSD, resulting in a reduction of the signal/background (S/B) value. Depending on the reaction conditions, the S/B value varies from 2.5 (enzyme catalysis with PS substrate at 0.5 mM) to 3.5 (HTS condition with PS at a concentration of 50 μM). Triton X-100 affects the fluorescence yield of a PS and PE mixture. The optimal concentration should be selected after trials of varying concentrations for the given PS substrate concentrations. The selection of optimal pH during the fluorescence detection of the DSB-3 adduct is also critical. The fluorescence yield of the DSB-3 adducts tends to increase with increasing pH, and a pH of >9.0 should be avoided.

In this report, we describe a second fluorescence assay for PSD catalysis, which uses 1,2-DAB/β-ME to make fluorescent adducts. Unlike DSB-3, 1,2-DAB/β-ME is widely available, has no autofluorescence in the presence of Triton X-100, which is required for PSD activity, and results in optimal fluorescence detection of PE. Furthermore, the 1,2-DAB/β-ME method does not make a fluorescent adduct with PS and produces a fluorescent adduct with a high signal/background ratio, thereby providing an improved and highly selective approach for inhibitor screening.

Results

1,2-DAB/β-ME reacts with PE to produce a fluorescent product

The work of Medici et al. (21) provided evidence that a mixture of 1,2-DAB/β-ME readily reacted with primary amines (e.g. ethanolamine), but not primary amines to which a carboxylate function was attached to the α-carbon (e.g. serine), to yield a fluorescent product. Based on this information, we examined the applicability of this reaction to the substrate (PS) and product (PE) of the PSD reaction. A schematic outline of the reaction strategy is shown in Fig. 1A, in which 1,2-DAB/β-ME is predicted to react with PE, but not PS. The data presented in Fig. 1B demonstrate that PE reaction with 1,2-DAB/β-ME yields a significant fluorescence signal that is not produced with other constituents of the enzyme reaction, either Triton X-100 or PS in combination with detergent.

Fig. 2 (A and B) shows the absorbance and fluorescence emission characteristics of the PE adduct, which has an absorption maximum at 364 nm. Fig. 2B demonstrates that the fluorescence emission maximum occurs at 425 nm when using excitation at 364 nm. The dependence of the fluorescence signal upon PE concentration is shown in Fig. 2C and demonstrates a reliable linear response with varying amounts of the lipid between 0 and 25 μM. The influence of different Triton X-100 concentrations varied in conjunction with varying PE concentrations is shown in Fig. 2D and indicates little variation in fluorescence signal up to 100 μM PE and a 3-fold range of Triton X-100, but the fluorescence signals over 100 μM PE require higher amounts of Triton X-100. Because 0.6% (9.3 mM) Triton X-100 ensures concentration-dependent fluorescence reading of higher PE without increasing background fluorescence, this detergent concentration was used for the remainder of experiments.

The 1,2-DAB/β-ME-based fluorescence assay can measure the activity of the purified PSD enzyme

The above parameters were used to monitor PSD reactions catalyzed by an affinity-purified, recombinant PSD enzyme (MBP-His₆-Δ34Pks PSD), encoded by the cDNA from Plasmodium knowlesi and expressed in E. coli, as described previously (19, 22). The fluorescence detection of the PSD reaction occurs in three steps. In the first step, the enzyme reaction is performed under optimal conditions for catalysis. In the second step, the catalytic reaction is arrested by adjusting the mixture to pH 9.85 with excess sodium tetraborate and then adding Triton X-100 to a final concentration of 9.3 mM and 1,2-DAB/β-ME to a final concentration of 1 mM/1.2 mM, respectively. Subsequently, the reaction mixture is incubated at 22°C for 1 h to enable fluorescent adduct formation. The data presented in Fig. 3A demonstrate the time-dependent production of adduct fluorescence from the PE product of the PSD reaction. Standard curves establishing PE adduct fluorescence as a function of phospholipid concentration (Fig. 3B) were generated and used to determine the molar amounts of PE formed, which are shown in Fig. 3C. The enzyme concentration dependence of the fluorescence intensity is shown in Fig. 3D and demonstrates a clear linear relationship between the amount of added enzyme and the fluorescence signal. The data in Fig. 3E show the linear dependence of the PE formation upon the amount of added Pks PSD. These data
establish the 1,2-DAB/β-ME is a sensitive and useful reagent for quantifying PE formation as a measure of PSD activity. Because many inhibitor screening assays are conducted at substrate concentrations close to the \( K_m \), we determined the kinetic constants for MBP-His\(_6\)-D\(_{34}\)KPSD using PE detection with 1,2-DAB/β-ME at substrate concentrations between 0 and 2 \( K_m \), and the results are presented in Fig. 4. Fig. 4A shows the \( V_0 \) versus [S] plot, and Fig. 4B shows the corresponding double reciprocal plot. The data for \( K_m \) (56.6 \( \mu M \)) and \( V_{max} \) (5.7 nmol/mg min) obtained using the fluorescence assay are comparable with values reported for MBP-His\(_6\)-Δ34KPSD determined using a radiochemical assay for catalysis (19).

**Modeling of the 1,2-DAB/β-ME derivatization reaction**

In their paper describing the 1,2-DAB/β-ME reaction with primary amines (21), Medici et al. did not provide a structure for the fluorescent adduct. To understand the chemistry of the derivatization of primary amines by 1,2-DAB/β-ME, we conducted model reactions using ethanolamine as the source of

---

**Figure 1.** 1,2-DAB/β-ME reacts with PE and produces a fluorescence signal. A, schematic depiction of the PSD reaction and PE product detection using 1,2-DAB/β-ME. B, fluorescence intensity after reaction of 1,2-DAB/β-ME with detergent alone or detergent micelles containing PS or detergent micelles containing PE. The phospholipids (50 \( \mu M \)) in 0.78 \( mM \) Triton X-100 concentration were mixed with an equal volume of 100 \( mM \) sodium tetraborate buffer (pH 9.85) containing 1,2-DAB (2 \( mM \)), 2.4 \( mM \) β-mercaptoethanol and incubated for 1 h at 22 °C in black-sided microtiter wells. Fluorescence was measured using a Tecan microplate reader with \( \lambda_{ex} = 355 \) nm and \( \lambda_{em} = 445 \) nm. The data are from four independent experiments, and values are means ± S.D. (error bars).

---

**Figure 2.** Optimization of PE adduct fluorescence detection. A, absorbance scan of 1,2-DAB adduct of PE, generated by incubation of 1,2-DAB and β-ME with PE for 1 h at room temperature, as described in the legend to Fig. 1B. Absorbance was monitored using a spectrophotometer (Beckman DU640). Absorbance was recorded at 5-nm wavelength intervals between 320 and 480 nm, and the values are shown after background correction using 1,2-DAB/β-ME in 0.78 \( mM \) Triton X-100. B, fluorescence emission scan of 1,2-DAB/β-ME–derivatized PE. Fluorescence intensities (F.I.) of the 1,2-DAB/β-ME adducts of PE at the various emission wavelengths were detected at 7-nm intervals, with an excitation wavelength of 364 nm. C, improved fluorescence signals can be obtained with optimized excitation (364 nm)/emission (425 nm) wavelengths and increased Triton X-100 (up to 1.55 \( mM \)) and PE (0–50 \( \mu M \)) in Triton X-100 were prepared as mixed micelles. The total phospholipid content of PE + PS was maintained at 50 \( \mu M \), and the ratio of PE/PS was varied with increasing PE content as shown. The fluorescence intensity was measured using a Tecan microplate reader with \( \lambda_{ex} = 364 \) nm and \( \lambda_{em} = 425 \) nm, after a 1-h incubation with 1,2-DAB/β-ME at 22 °C. The data are from four independent experiments and are means ± S.D. D, fluorescent adduct formation of 1,2-DAB/β-ME with higher concentrations of PE requires higher amounts of Triton X-100. PE and PS (200 \( \mu M \) in 0.78 \( mM \) Triton X-100) were prepared as mixed micelles. The total phospholipid content of PE + PS was maintained at 200 \( \mu M \) and the ratio of PE/PS was varied with increasing PE content as shown. Various final concentrations of Triton X-100 were added into the fluorescence detection solution. Fluorescence intensity was measured using a Tecan microplate reader with \( \lambda_{ex} = 364 \) nm and \( \lambda_{em} = 425 \) nm, after a 1-h incubation at room with 1,2-DAB/β-ME. The data are from four independent experiments and are means ± S.D. (error bars).
primary amine, HPLC analysis, and high-resolution MS analyses of the resultant products, as shown in Fig. 5. The two most abundant fractions, A and B, were identified and separated by HPLC analysis (Fig. 5A). Only fraction B fluoresced under long-wave UV light, indicating that the fluorescent reaction products are included in fraction B. The high-resolution mass
spectrometric analyses of the fractions A and B are shown in Fig. 5, B and C, respectively. The two most prominent species from fraction B were initially observed as the major products of the model reaction. One was found to be C_{14}H_{18}NO_{2}S^{+} (m/z 264.105) by high-resolution mass spectrometric analysis, consistent with attachment of a single β-mercaptoethanol and ethanolamine to 1,2-DAB to form a oxathiolane (compound IV in Fig. 6). The other was found to be C_{16}H_{24}NO_{3}S_{2}^{+} (m/z 342.119), consistent with the formation of an iso-indole oxathiolane with an additional β-mercaptoethanol (compound V in Fig. 6). Collision-induced decomposition of compound V yielded major product ions at m/z 264.092, consistent with compound IV (C_{14}H_{18} NO_{2}S^{+}), and at m/z 174.091, as shown in Fig. 5D and Fig. S1.

**Application of 1,2-DAB/β-ME method to detect PSD activities in cell extracts and membrane preparations from various cell types**

To test the broader applicability of this new PSD assay, we also examined activity using cruder preparations of enzyme. In Fig. 7A, we show the catalytic activity of PSD in crude lysate preparations from *E. coli*, and the data demonstrate that these preparations produce a strong fluorescence signal for catalysis that increases linearly with time. The rate of catalysis in Fig. 7A corresponds to 0.74 μmol/h/mg of cell extract protein and is shown graphically in Fig. 7B. Fig. 7C shows the time-dependent activity of enzyme obtained from purified bacterial membranes isolated by ultracentrifugation, using 1.4 μg of protein per 100 μl of reaction volume. Fig. 7D demonstrates the dependence of the reaction upon membrane protein concentration. Fig. 8 shows a comparison between *E. coli* WT and *psd* strains grown at permissive (30 °C) and nonpermissive (42 °C) temperatures. Even at the 30 °C permissive temperature, the *psd* enzyme is quite labile, and at 42 °C, *psd* catalysis is ~4.2% of that observed for WT enzyme.

We also tested mitochondrial preparations from the yeast, *Candida albicans* (Fig. 9), and this organelle fraction produces a strong fluorescent signal for catalysis. The fluorescence signal is entirely PSD-dependent because there is no gain of fluorescence emission when PS substrate is not added to the reaction (Fig. S2). Finally, we tested mitochondrial fractions from HeLa cells, as shown in Fig. 10 (A and B), and these preparations generate a significant signal for the activity of PSD, although it is...
Fluorescence detection of PS decarboxylase activity

considerably weaker than values obtained for bacteria or C. albicans. To further verify the accuracy and reliability of the 1,2-DAB/β-ME–based enzyme assay, we monitored PE formation using TLC analysis of lipid components extracted from the PSD enzyme reactions of HeLa mitochondrial fractions. Fig. 10 shows that quantification of PE made from the PS substrate by the PSD reaction was very similar in the 1,2-DAB/β-ME analysis and comparable with TLC analysis and phosphate quantification of the PSD reaction products (Fig. 10D). The data confirm that the 1,2-DAB/β-ME–based PSD assay is very accurate for measuring PSD activities. In summary, the data from Figs. 7–10 provide clear evidence that the PE generated by catalysis from diverse sources of PSD enzymes of varying purity is reliably detected by the 1,2-DAB/β-ME method.

Fluorescent detection of PSD activity is amenable to HTS platforms

We also evaluated the variability of the 1,2-DAB/β-ME assay system using 96-well and 384-well formats. Mean and S.D. of the samples were used to calculate S/B, coefficient of variation (CV), and Z' factors to ensure the assay quality (23). We
observed 1.3% CV for positive control reaction samples with active PSD enzymes and 1% CV for negative control samples with heat-inactivated PSD enzymes and S/B = 24.3 and a $Z'' = 0.96$, demonstrating low variability and high robustness of the assay.

Discussion

Phospholipid metabolism is a fundamental requirement for membrane biogenesis across all biological domains (Archaea, Bacteria, and Eukarya). Despite their essential role in cellular and organismal survival, the pathways for the synthesis of phospholipids have not been the subject of seriously focused pharmacological attacks, for the purpose of identifying and creating new types of inhibitors that might be especially useful as antimicrobial or antineoplastic agents. Historically, a major impediment to discovery of such inhibitors has been the relatively complicated nature of assays for phospholipid synthetic enzymes. These difficulties are bipartite, with one problem being the availability of purified enzymes, of sufficient quantity and activity, and a second problem being the nature of assays to easily detect these enzyme activities. In this report and in a recent publication (19), we have addressed and simplified the problem of measuring PSDs. Prior to the assays described in these two papers, the most streamlined and accurate procedure for measuring PSDs involved 1) preparing membrane extracts from organisms, 2) incubating the membranes with detergent micelles containing radioactive [1$\text{C}$]PS in a gas-tight vessel, 3) trapping evolved $^{14}$CO$_2$ on KOH-saturated filter paper, and 4) quantifying the trapped $^{14}$CO$_2$ by liquid scintillation spectrometry. More involved procedures using $[^3]$HPS or fluorescent PS as substrates, followed by lipid extraction of enzyme reactions, and subsequently analysis of thin-layer chromatograms have also been used, but these are even more labor-intensive and time-consuming than the $^{14}$CO$_2$-trapping method. The methods described in this paper use the sequential addition of reagents to a single well of multiwell plates to develop strong fluorescence signals that accurately report enzyme activity.
Although this report is focused upon PSD, it highlights an approach that is more generally applicable to broadly probing phospholipid and sphingolipid metabolism for novel inhibitors. To facilitate our studies, we have first taken advantage of a variant recombinant parasite enzyme (MBP-His<sub>6</sub>-34PkPSD) expressed at high levels in <i>E. coli</i> and then affinity-purified (24). Certainly, the heterologous overexpression of enzymes in either bacteria or yeast or baculovirus-infected insect cells is now commonplace. However, in the case of PSD, our experience with different heterologous systems for overexpression enabled us to fine-tune the production of PkPSD to yield a soluble and stable version of an otherwise membrane-bound and relatively unstable enzyme. This abundant source of enzyme allowed us to freely test different approaches to developing a high-sensitivity fluorescence assay for PSD. Although we began these studies with a purified PSD, it is clear that the assay system we developed is robust and sensitive enough to detect endogenous levels of PSD in <i>E. coli</i> cell-free extracts, <i>E. coli</i> membranes, yeast mitochondria, and mammalian mitochondria isolated from a tissue culture cancer cell line, as shown in Figs. 7–10. When crude protein fractions were used as sources of PSD, nascent PE and compounds containing primary amine groups inevitably interacted with 1,2-DAB/\(\beta\)-ME, resulting in higher fluorescence background and lowering S/B values. Still, the S/B values were respectable, in the range of 5.8–9 among the various crude PSD sources. Another problem of using crude protein fractions in the PSD assay is how we can identify PE synthesized by the PSD but not the PE generated through the non-PSD pathways. This can be a concern when the PSD assay depends on the fluorescence detection of PE. The 1,2-DAB/\(\beta\)-ME–based PSD assay, however, excludes PE formed from the precursors, such as lyso-PE, Etn, phospho-Etn, or CDP-Etn, because excess 1,2-DAB/\(\beta\)-ME present in the assay will make a fluorescent adduct with the precursors as well as the PE. Therefore, there will be no increase of fluorescence by a non-PSD pathway. Only PE formed from the PS substrate results in the increase of fluorescence. In Fig. 10 (C and D), we verified the accuracy of the 1,2-DAB/\(\beta\)-ME–based PSD assay of HeLa cell mitochondria with the TLC/lipid phosphorus analysis of lipids that were extracted from the PSD reactions. The data also revealed that the net gain of PE (10.9 ± 0.7%) after a 1-h PSD assay was matched with the net loss of PS (10.2 ± 1.7%), whereas the sum of the two lipids did not change much (from 85.5 ± 3% at 0 min to 86.1 ± 4.1% at 60 min). The data indicate that PE was not generated from other PE synthetic pathways but indeed generated from PSD activity using a substrate, PS.

We recently reported a similar assay for PSD catalysis using the bis-aldehyde reagent, DSB-3. The newly developed assay applying 1,2-DAB/\(\beta\)-ME to generate a fluorescent PE adduct provides some significant advantages over the DSB-3 method. Most notably, the 1,2-DAB/\(\beta\)-ME reagent does not form a fluorescent adduct with the PS substrate, whereas DSB-3 forms adducts with both PS and PE, albeit with different fluorescence yields of the products. The most important consequence of the difference in selectivity between the two approaches is that the...
signal/background ratio of the 1,2-DAB/β-ME method is ~24, whereas that for DSB-3 is 3.5. Thus, the dynamic range for detecting PSD inhibition in screening assays is significantly improved with the 1,2-DAB/β-ME reagent. Moreover, the 1,2-DAB/β-ME method produces a negligible background signal following incubation with 0.5 mM PS substrate (see Fig. 1B). This new assay system for PSDs now makes it relatively simple to screen for inhibitors of PSDs from pathogenic yeasts and bacteria and neoplastic cells and tissues.

**Experimental procedures**

**Materials**

All chemicals for bacterial and yeast growth media were purchased from Sigma, Fisher Scientific, and Difco. Phospholipids were purchased from Avanti Polar Lipids. Reagents for quantifying protein were from Bio-Rad. 1,2-DAB and PS were obtained from Sigma, Fisher Scientific, and Difco. Phospholipids were identified by polyacrylamide SDS-gel electrophoresis, followed by Coomassie staining of the gel and Western blotting analysis using anti-His6 antibody.

**Expression and purification of MBP-His$_6$-Δ34PkPSD**

Expression of MBP-His$_6$-Δ34PkPSD in E. coli was performed as described previously (24). A Rosetta DE3 strain harboring a pMAL-c2x-His$_6$-Δ34PkPSD plasmid vector was grown to saturation overnight in 1 liter of lysogeny broth medium with 0.2% glucose, ampicillin (100 μg/ml), and chloramphenicol (34 μg/ml) and then diluted 100-fold and grown to A$_{600}$ ~0.5 at 37 °C. Expression of MBP-His$_6$-Δ34PkPSD was induced by the addition of 0.3 mM isopropylthioligalactoside for 2 h at 37 °C. The cells were harvested by centrifugation (4,000 g, 20 min, 4 °C) and washed by resuspension in water and recentrifugation. The cells were resuspended in 25 ml of the disruption buffer (20 mM potassium phosphate, pH 7.4, 200 mM NaCl, 1 mM EDTA, and 10 mM β-ME, flash-frozen in a dry ice-ethanol bath, stored overnight at −20 °C, and subsequently thawed on ice water. Cell extracts were obtained by sonication (15-s burst at 30% amplitude using a Fisher Sonic Dismembrator 500, performed eight times, interrupted by 30-s cooling intervals (using an ice water bath), followed by centrifugation at 20,000 g × 20 min, at 4 °C. MBP-His$_6$-Δ34PkPSD was purified from the resultant supernatants by amylose column affinity chromatography using methods described in the instruction manual from New England Biolabs (catalog no. E8200S). Briefly, the cell extracts were further diluted 5-fold in disruption buffer and applied to an amylose affinity column (~10 ml). The column was washed with 6-ml aliquots of the disruption buffer 11 times. MBP-His$_6$-Δ34PkPSD proteins were eluted with the disruption buffer containing 10 mM maltose. 20 fractions of 1.2 ml each were collected. The fractions containing the MBP-His$_6$-Δ34PkPSD proteins were identified by polyacrylamide SDS-gel electrophoresis, followed by Coomassie staining of the gel and Western blotting analysis using anti-His$_6$ antibody.

**Fluorescence-based PSD assay**

The initial fluorescence-based PSD assays were conducted in a 100-μl volume in a microcentrifuge tube with 0.5 mM PS substrate prepared as detergent micelles in 1.55 mM Triton X-100 and varying amounts (0–1.25 μg) of MBP-His$_6$-Δ34PkPSDs in a buffer of 50 mM NaCl and 10 mM potassium phosphate, pH 7.4. The enzyme reaction was conducted at 30 °C for the indicated times, with shaking at 120 rpm, and terminated by the addition of 25 μl of 100 mM sodium tetraborate buffer, pH 9.85. The reaction samples were snap-frozen on dry ice and kept at −30 °C, until further processing for fluorescence detection. After thawing, a 37.5-μl aliquot of the reaction sample was added to a black-sided microtiter well of a 96-well microtiter plate (Corning, catalog no. 3631) containing 82.5 μl of fluorescence detection buffer (2 μl of 61 mM 1,2-DAB, 2 μl of 75 mM β-ME, 53.2 μl of 100 mM sodium tetraborate buffer, pH 9.85, and 18.4 μl of 1 mM potassium phosphate buffer, pH 7.4, and 6.9 μl of 15 mM Triton X-100), and the reaction solution was incubated at 22 °C with shaking at 100 rpm under reduced light conditions. Fluorescence intensity was measured using a Tecan Infinite M1000 microplate reader, which was managed by the software program, i-control (Tecan Group Ltd.). To measure PSD activities from the fluorescence emission data, a standard curve was generated by conducting mock PSD enzyme reactions with heat-inactivated enzyme and various ratios of PE/PS mixed micelles, in which the total phospholipid content of the micelles was maintained at 0.5 mM. These mock reactions were performed and diluted for fluorescence detection in the same way as the PSD reactions. Fluorescence intensities from the mock reactions were used to produce the standard curve of fluorescence emissions for defined PE concentrations in the mixed PE/PS micelles.

**PSD enzyme kinetics**

Kinetic parameters were determined using both a radiochemical assay (8) and the fluorescence method for measuring PSD activity. Affinity-purified MBP-His$_6$-Δ34PkPSD was prepared as described above and used for both methods (19). The 1,2-DAB-based fluorescence assays contained varied concentrations of PE (0-100 μM), 0.78 mM Triton X-100, and 31.3 ng of purified MBP-His$_6$-Δ34PkPSD in a 62.5-μl reaction volume. After incubation at 37 °C for 20 min, the reaction tubes were shifted to 0 °C. Standard curves of product formation were generated from mixtures of PE and PS as in Fig. 2C. Mock enzyme reactions contained heat-inactivated enzyme. Reactions were arrested by the addition of 7.5 μl of 100 mM sodium tetraborate buffer, pH 9.85, and fluorescence detection was initiated by the addition of 55 μl of buffer (2 μl of 61 mM 1,2-DAB, 2 μl of 75 mM β-ME, 43.8 μl of 100 mM sodium tetraborate buffer, pH 9.85, and 7.2 μl of 15 mM Triton X-100) to the PSD assay solution. 1,2-DAB/β-ME adduct formation was allowed to proceed for 1 h at room temperature with shaking at 100 rpm. Enzyme velocity is reported as nmol/μg of protein min. The $K_m$ value and the $V_{max}$ were determined using GraphPad Prism version 8 software.

**Fluorescence-based PSD assay in HTS platform**

The assay was conducted as described above in the PSD enzyme kinetics method with some modifications. In the first step, the PSD assay was conducted in a 60-μl assay volume containing 2 ng/μl purified MBP-His$_6$-Δ34PkPSD and 50 μM PS.
substrate at 30 °C for 45 min in a 96-well microtiter plate (Corning, catalog no. 3631). In the second step, PSD inactivation and fluorescence adduct formation were performed by the addition of 60 μl of buffer containing 2 μl of 61 mM 1,2-DAB, 2 μl of 75 mM β-ME, 49.1 μl of 100 mM sodium tetraborate buffer, pH 9.85, and 6.9 μl of 15 mM Triton X-100). In the third step, fluorescence intensity was measured using a TECAN Infinite M1000 microplate reader as described above.

**Fluorescence detection of PS decarboxylase activity**

To determine the structure of the primary amine adduct after treatment with 1,2-DAB/β-ME, 0.5 mM ethanolamine was incubated with the 1 mM 1,2-DAB and 1.2 mM β-ME in 100 mM sodium tetraborate buffer (pH 9.85) in a 1-ml volume. The reaction proceeded for 30 min at room temperature. A 200-μl aliquot of the reaction mixture was injected into an HPLC column as described below, and five fractions with a distinct UV absorbance peak were collected. A fraction with strong fluorescence characteristics was identified using a handheld UV light source, and the fraction subsequently underwent further mass spectral analysis. MS was performed using a Synapt quadrupole TOF mass spectrometer (QqTOF) (Waters Associates, Milford, MA) in the high-resolution double reflector mode as described below. Mass assignments were measured at 2 ppm error after calibration.

**HPLC separation**

Chromatographic separations were performed on a Waters Acquity Binary Solvent Manager inlet system (Waters, Manchester, UK). The reaction products were injected onto a reversed-phase silica HPLC column (Kinetex, 5 μm C18 100 Å, 150 × 2.1 mm) and subjected to gradient elution using mobile phase A (aqueous ammonium acetate, pH 5.7) and mobile phase B (methanol) (25). The starting HPLC solvent consisted of 80% Solvent A and 20% Solvent B at a flow rate of 0.3 ml/min. From 0 to 1 min, the concentration of Solvent B was increased to 25%. For 24 min, the concentration of Solvent B was linearly increased to 75% and then increased to 80% over 5 min. The concentration of Solvent B was then increased to 95% over 3 min before recycling to 20% Solvent B over 12 min. The effluent was monitored using a photodiode array detector (Shimadzu SPD-M10AVP diode array detector), which permitted direct recording of the UV absorbance spectra from 210 to 500 nm during sample elution. A chromatogram corresponding to absorbance at 365 nm was reconstructed to indicate elution of reaction products. Fractions (1 min) were collected in test tubes and tested for the presence of fluorescent compounds eluting from the column using a model UVL-21 handheld UV lamp with an emission wavelength of 366 nm (Ultra-Violet Products, Inc., San Gabriel, CA).

**MS**

MS was carried out using a Synapt G2-S instrument (Waters) in the positive ion mode (tandem quadrupole TOF mass spectrometer). Fractions collected from the HPLC separation at 8.6 and 9.6 min were infused directly into the electrospray ion source without further modification of the HPLC effluent system. Mass spectrometer parameters included the following: electrospray ionization voltage, 2,500 V; sampling cone, 40 V; source offset, 80 V; source temperature, 80°C; desolvation temperature, 150°C; desolvation gas, 500 liters/h; nebulizer, 6.0 bar. Collision-induced dissociation was carried out with argon as the collision gas and a collision energy of either 25 or 40 V, as indicated. Mass spectra were recorded in “high-resolution” mode.

**Structural determination of 1,2-DAB/β-ME-ethanolamine**

To determine the structure of the primary amine adduct after treatment with 1,2-DAB/β-ME, ethanolamine was obtained from a Rosetta DE3 strain as described previously (19). The PSD enzyme assay was conducted in a 100-μl volume and contained E. coli cell-free extracts (200 ng/μl) or E. coli membrane fractions (14.1 ng/μl), 0.5 mM PS, and 3.1 mM Triton X-100. The reaction was performed at 37 °C for 0–45 min. Fluorescence detection of the PSD activity was performed as described for the purified enzyme. A standard curve for calculation of PSD activity was generated by conducting mock PSD enzyme reactions with heat-inactivated E. coli cell extracts or membrane fractions and various ratios of PE/PS mixed micelles. S/B values for the E. coli cell-free extracts and membrane fractions were 6.8 ± 0.7 and 27.5 ± 1.8, respectively (mean ± S.D.). To compare PSD activity between WT and the psd mutant strain, HB101 (WT) and EH150 (psd mutant strain) (1) were initially grown at 30°C to early log phase and then maintained at 30°C or shifted to 42°C for 4 generations. The enzyme assays were performed at 30°C.

**Fluorescence-based PSD assay of E. coli cell extracts and membranes**

E. coli cell-free extracts and membrane fractions were obtained from a Rosetta DE3 strain as described previously (19). The PSD enzyme assay was conducted in a 100-μl volume and contained E. coli cell-free extracts (200 ng/μl) or E. coli membrane fractions (14.1 ng/μl), 0.5 mM PS, and 3.1 mM Triton X-100. The reaction was performed at 37 °C for 0–45 min. Fluorescence detection of the PSD activity was performed as described for the purified enzyme. A standard curve for calculation of PSD activity was generated by conducting mock PSD enzyme reactions with heat-inactivated E. coli cell extracts or membrane fractions and various ratios of PE/PS mixed micelles. S/B values for the E. coli cell-free extracts and membrane fractions were 6.8 ± 0.7 and 27.5 ± 1.8, respectively (mean ± S.D.). To compare PSD activity between WT and the psd mutant strain, HB101 (WT) and EH150 (psd mutant strain) (1) were initially grown at 30°C to early log phase and then maintained at 30°C or shifted to 42°C for 4 generations. The enzyme assays were performed at 30°C.

**Fluorescence-based PSD assay of C. albicans PSDs**

To assess PSD activities in a pathogenic yeast, mitochondrial fractions were purified from a C. albicans strain, SC5314 (26). Growth of the yeast strains and preparation of the mitochondria were as described previously (27, 28). Briefly, yeast strains were grown to early log phase in 1-liter cultures of YP-lactate medium supplemented with ethanolamine (2 mM) at 30°C. Crude mitochondria were prepared by Dounce homogenization of spheroplasts generated by zymolyase treatment of the cells, followed by differential centrifugation (29). The mitochondrial pellets were suspended in 0.6 m sorbitol, 20 mM MES, pH 6.0, and used as the enzyme source. PSD reactions in 100 μl contained mitochondrial fractions (8 μg of protein), 0.5 mM PS, and 3.1 mM Triton X-100 and were performed at 30°C, for 0–60 min. Fluorescence detection of PSD activity was performed as described above. A standard curve for calculation of PSD activity was generated by conducting mock PSD enzyme reactions with heat-inactivated mitochondrial fractions. S/B value for the given assay condition was 7.6 ± 0.8 (mean ± S.D.).

**Fluorescence-based assay of HeLa cell PSDs**

Mitochondrial fractions were purified from HeLa Ohio cells. The cells were grown to 85% confluency in 10 culture dishes of 150-mm diameter, each containing 15-18 ml of Dulbecco’s modified Eagle’s medium/F-12 (1:1). Cells were harvested by scraping with a squeegee and washed twice with PBS buffer, by centrifugation at 600 × g for 5 min and resuspended in 5 ml of...
Fluorescence detection of PS decarboxylase activity

References


Funding and additional information—This work was supported by National Institutes of Health Grants AI097218 (to C. B. M.) and GM104485 (to D. R. V.) and by National Institutes of Health Grants AI123321 and AI138139 (to C. B. M.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: PE, phosphatidylethanolamine; 1,2-DAB, 1,2-diacyetyl benzene; β-ME, β-mercaptoethanol; DSB-3, distyrylbenzene-bis-aldehyde (4,4’-(1E,1’E)-(2,5-bis((2,5,8,12,15,18-hexaoxanadecan-10-yl)oxy)-1,4-phenylene) bis (ethene-2,1-diyl) dibenzaldehyde); HTS, high-throughput screen; LACTB, serine β-lactamase-like protein; MBP, maltose-binding protein; Pk, Plasmodium knowlesi; PSD, phosphatidylserine decarboxylase; S/B, signal/background; CV, coefficient of variation.

Acknowledgments—We thank Denton Hoyer and Mark Plummer (Yale Center for Molecular Discovery (YCMD)) for insightful input on the structure model.

Data availability

All data described are contained within the article and supporting information.
Fluorescence detection of PS decarboxylase activity


