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Streptomyces Phospholipase D Mutants with Altered Substrate Specificity Capable of Phosphatidylinositol Synthesis

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The substrate specificity of a phospholipase D (PLD) from Streptomyces antibioticus *was altered by site-directed saturation mutagenesis, so that it was able to synthesize phosphatidylinositol (PI). Mutations were introduced in the* pld *gene at the positions corresponding to three amino acid residues that might be involved in substrate recognition, and the mutated genes were expressed in* Escherichia coli *BL21 (DE3). High-throughput screening of approximately 10000 colonies for PI-synthesizing activity identified 25 PI-synthesizing mutant PLDs. One of these mutant enzymes was chosen for further analysis. The structure of the PI synthesized with the mutant enzyme was analyzed by HPLC-MS and NMR. It was found that the mutant enzyme generated a mixture of structural isomers of PIs with the phosphatidyl groups connected at different positions of the inositol ring. The phosphatidylcholine-hydrolyzing activity of the mutant PLD was much lower than that of the wild-type enzyme. The mutant enzyme was able to transphosphatidylate various cyclohexanols with a preference for bulkier compounds. This is the first example of alteration of the substrate specificity of PLD and of PI synthesis by* Streptomyces *PLD.*

Introduction

Phospholipase D (PLD, E.C. 3.1.4.4.) is an enzyme that hydrolyzes phospholipids into phosphatidic acid (PA) and the corresponding alcohols. PLD also catalyzes transphosphatidylation, in which the polar head groups of phospholipids are exchanged with the coexisting alcohol. By exploiting this activity, various phospholipids can be synthesized enzymatically from naturally abundant phospholipids such as phosphatidylcholine (PC) or lecithin.

PLDs from microorganisms such as *Streptomyces* are widely used for industrial phospholipid syntheses, because of their broad substrate specificity toward alcohol compounds as well as the ease of enzyme preparation. Most natural types of phospholipids [i.e., PC, phosphatidylethanolamine (PE), phosphatidylserine (PS), or phosphatidylglycerol (PG)] can be synthesized from commercially available lecithin and appropriate acceptor alcohols by using *Streptomyces* PLD.[1] However, no *Streptomyces* PLD is capable of synthesizing phosphatidylinositol (PI) from lecithin and *myo*-inositol, possibly because of steric hindrance towards the bulky inositol molecule in the substrate binding pocket, and also because of relatively lower activity towards secondary alcohols than primary alcohols, as pointed out by D'Arrigo et al.[1b,c] Although some PLDs with plant origins[2] are reported to catalyze PI synthesis, these enzymes are not easily available for industrial purposes.

PI is now attracting attention because it has certain biological effects.[3] For example, Sparks's research group reported that soybean PI stimulated a reverse cholesterol transport pathway in rats.[3a,b] They also reported that orally administered PI increased high-density lipoprotein cholesterol levels in humans;[3c] this illustrates the therapeutic value of PI. In addition, Yanagita reported that dietary PI can lower the levels of triacyl glycerol in the serum and liver of mice.[3d]

Currently, PI is produced industrially by extraction from natural sources such as soybean lecithin. However, the PI contents of such natural sources are not very high (approximately 15% in soybean phospholipids),[4] and the extraction processes require large amounts of solvent. Furthermore, PI extracted from natural sources is a heterogeneous mixture of PI molecular species with different fatty acid residues, which might be problematic if the PI is intended for use as a fine chemical. There-

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fore, the PLD-mediated process is promising as an alternative for PI production. Here we describe the creation of mutated *S. antibioticus* PLD (Sa-PLD) with PI-synthesizing activity by site-directed saturation mutagenesis.

Results

Mutation strategy

The strategy employed in this study for altering the enzyme's specificity was to modify the substrate binding pocket, so that the bulky acceptor (i.e., *myo*-inositol) can access the active site. Figure 1 shows a structure model of the substrate binding pocket of Sa-PLD in complexation with dihexanoylphosphatidylcholine (diC6PC). This model was constructed from two tertiary structures: the ligand-free wild-type (WT) Sa-PLD (structure 1, PDB code: 2ZE4) and its inactive H168A mutant in complexation with diC6PC (structure 2, PDB code: 2ZE9). The diC6PC portion in structure 2 was superimposed onto structure 1. The model structure thus constructed is very close to that of the phosphatidyl-enzyme intermediate in which the dibutyrylphosphatidyl moiety is covalently bound to PLD from *Streptomyces* sp. PMF (PMF-PLD).[5] The head group choline moiety and portions of acyl groups were not visible, due to poor electric density in structure 2.

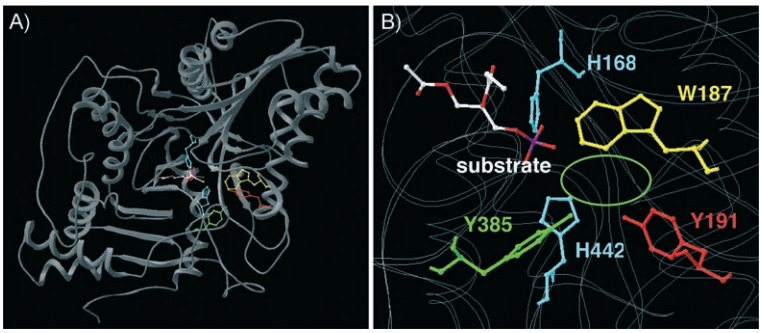


Figure 1. Structure model of Sa-PLD complexed with the substrate. A) Overview. B) Substrate binding site. The model was drawn with the Swiss PDB viewer. The substrate is colored in CPK mode (carbon, white; oxygen, red; phosphorus, purple). The catalytic histidine residues are colored in blue, W187 in yellow, Y191 in red, and Y385 in green. The green oval indicates the space that was expected to accommodate the head groups and the acceptor.

According to the reaction mechanism proposed for PMF-PLD,[5a] H170 attacks the phosphorus atom to form the phosphatidyl-enzyme intermediate (first step), followed by cleavage of the intermediate by water (hydrolysis) or alcohol (transphosphatidylation; second step). Both reaction steps are considered to be S_N2-type nucleophilic substitutions with in-line attacks of the phosphorus atom by the nucleophiles from the side opposite to the leaving group. Taking the proposed reaction mechanism into consideration, we estimated that the polar head group, which was invisible in the structure model of Sa-PLD (Figure 1B), might be positioned at the opposite side of the H168 with the phosphorus atom in-between. We also reckoned that the second nucleophile (i.e., water or alcohol) should arrive from the opposite side of the H168 to attack the phosphorus atom of the phosphatidyl-enzyme intermediate. On the basis of the above estimation, we found that a space surrounded by W187, Y191, and Y385 should be suitable to accommodate the head group moiety or the acceptor compound. It therefore seemed reasonable to alter this space by modifying these three residues so that a bulky *myo*-inositol molecule could enter, and so we constructed the mutated *pld* gene library by introducing mutations into the three positions.

Screening for PI-synthesizing mutants

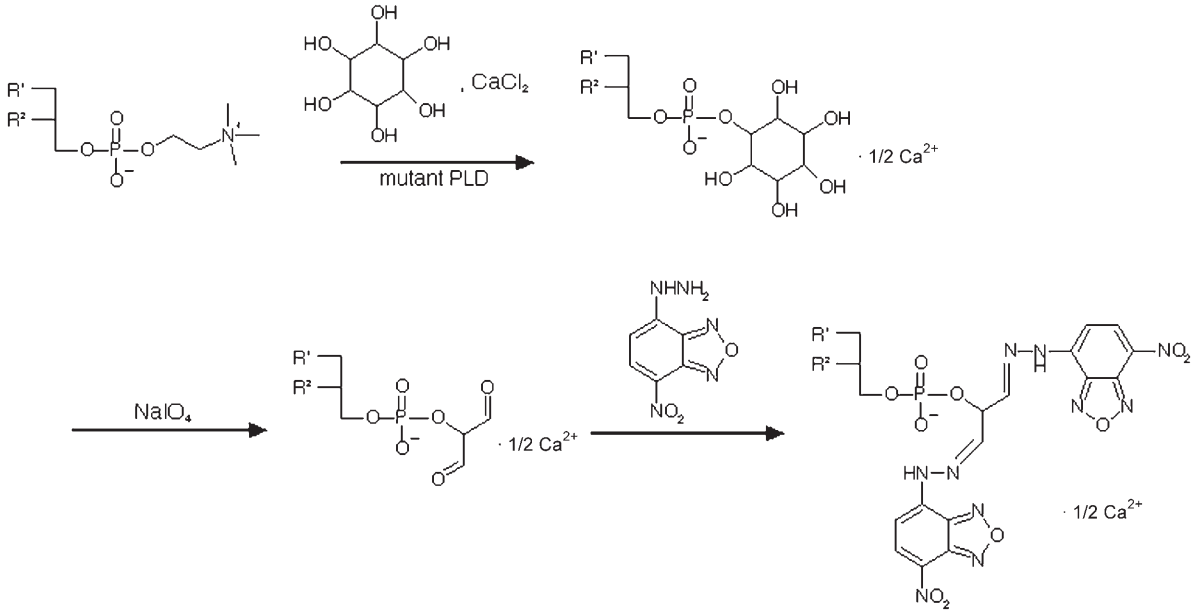
Screening of approximately 10000 colonies for PI-synthesizing activity by the high-throughput method (Scheme 1) identified 48 positive clones. DNA sequencing of those positive clones revealed that some of them had the same mutations, resulting in the identification of 29 different mutants. Each of the 29 mutant enzymes was prepared from a liquid culture, and its PI-synthesizing activity was tested with dioleoylphosphatidylcholine (DOPC) and *myo*-inositol as the substrates. Out of the 29 clones, four were found to be pseudopositive (i.e., negative), and finally 25 were identified as independent PI-synthesizing mutants (see the Supporting Information). The mutated amino acid sequences of the mutants were various, and we could not find any common rule for the mutation pattern for the PI-synthesizing activity. Out of the 25, we selected one of the mutants (187F/191R/385Y, or FRY) for the subsequent experiments.

Structure determination of PI synthesized by FRY

Figure 2A demonstrates a TLC separation of the products of the PI synthesis reaction by the FRY mutant. Two spots with R_f values of 0.39 and 0.32 were observed. After a 12 h reaction time, the upper and lower spots corresponded to 11% and 21% of the total phospholipids, respectively. Since *myo*-inositol has six hydroxy groups, which are not chemically equivalent, there are six possible structures for the enzymatically synthesized PIs (Figure 2B). We thus expected that the two spots might be structural isomers of PI with the phosphatidyl group connected at different positions of the inositol ring.

The lipids corresponding to the two spots were purified by silica gel column chromatography (Figure 2A, lanes 2 and 3). From 180 mg (0.23 mmol) of DOPC, 2.0 mg (0.0023 mmol) of the upper spot compound and 2.5 mg (0.0029 mmol) of the lower spot compound were obtained. The isolation yields were 1% (upper spot) and 1.2% (lower spot). ^{1}H NMR analyses of the two compounds revealed that the lower spot was 1-PI and/or 3-PI, and the upper spot was 4-PI and/or 6-PI (Supporting Information).

Figure 2C shows HPLC-MS chromatograms of the chemically synthesized authentic PI and the reaction products. The PI isomers elute in the following order: 4(6)-PI, 5-PI, 2-PI, and 1(3)-PI (Supporting Information). Analysis of the enzymatic reaction



Scheme 1. Principle of the high-throughput screening. PI generated by the enzymatic reaction was oxidized with periodate to give an aldehyde-containing lipid, which was then coupled with NBDH. Note that the chemical structures of the aldehyde-containing lipid and its NBD derivative were not determined.

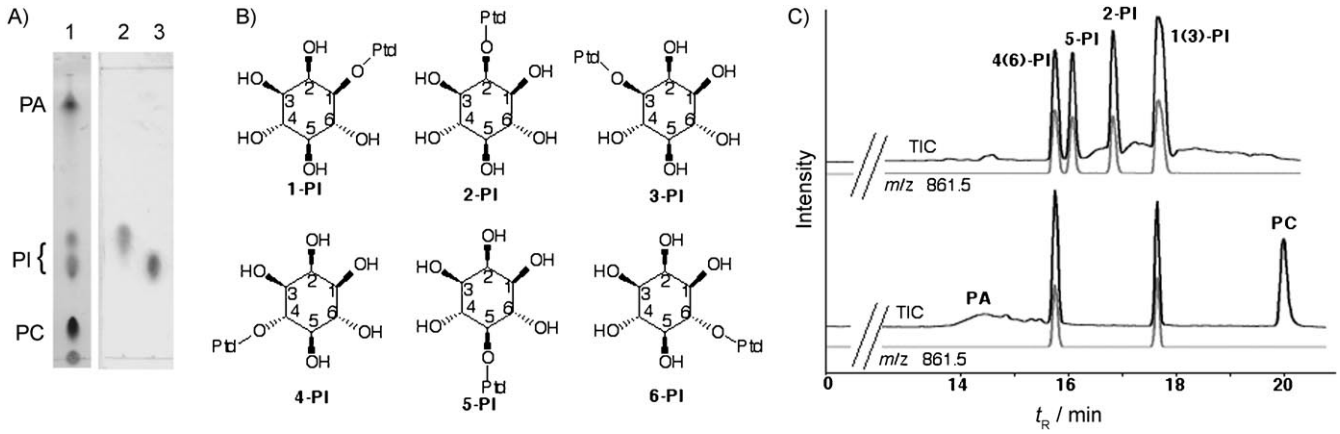


Figure 2. Identification of the PI isomers synthesized with FRY. A) TLC separation of the phospholipids. From the enzymatic reaction products (lane 1), the two PI isomers were purified by silica gel column chromatography (lanes 2 and 3). B) The possible structures of PI isomers. "Ptd" indicates the dioleoylphosphatidyl group. C) HPLC-APCI-MS separation profiles of chemically synthesized PI isomers (upper), and the enzymatic reaction products (lower). Total ion chromatograms (TIC) and extracted ion chromatograms (EIC) at *m/z* of 861.5 are shown. Note that the peak intensities might not exactly represent the actual phospholipid composition, due to differences in ionization efficiencies.

products by the HPLC method revealed two peaks with *m/z* values of 861.5 ($[M-H]^-$), confirming that both the peaks were structural isomers of dioleoyl-PI ($M_W = 862.6$). The more rapidly eluting peak (corresponding to the upper spot on TLC) had the same retention time as the standard 4(6)-PI, while the slower peak (the lower spot on TLC) had the same retention time as the standard 1(3)-PI. The HPLC result confirmed again that the FRY mutant had generated 1(3)-PI and 4(6)-PI, but not 2-PI and 5-PI.

We could not distinguish 1-PI from 3-PI or 4-PI from 6-PI because the structural symmetry of the *myo*-inositol moiety hindered the separations of these isomers by the HPLC method, and because the NMR analysis was unable to distinguish the symmetrical structure in its intact form.

Comparison of WT and FRY

The WT and FRY enzymes were purified to homogeneity (Figure 3A) and used for kinetic analyses. Figure 3B and C show plots of PLD activity versus substrate concentration for WT and FRY, respectively, in DOPC hydrolysis. In both cases, the plots show sigmoidal curves. These kinetic profiles are due to the physical state of the substrate, and it has been suggested that these sigmoidal curves fit the Hill model much better than they do the Michaelis–Menten equation.[6] We therefore applied the Hill equation and calculated the K_M and V_{max} values. The K_M values of both enzymes were similar, whereas FRY showed a much lower V_{max} than the WT (Table 1).

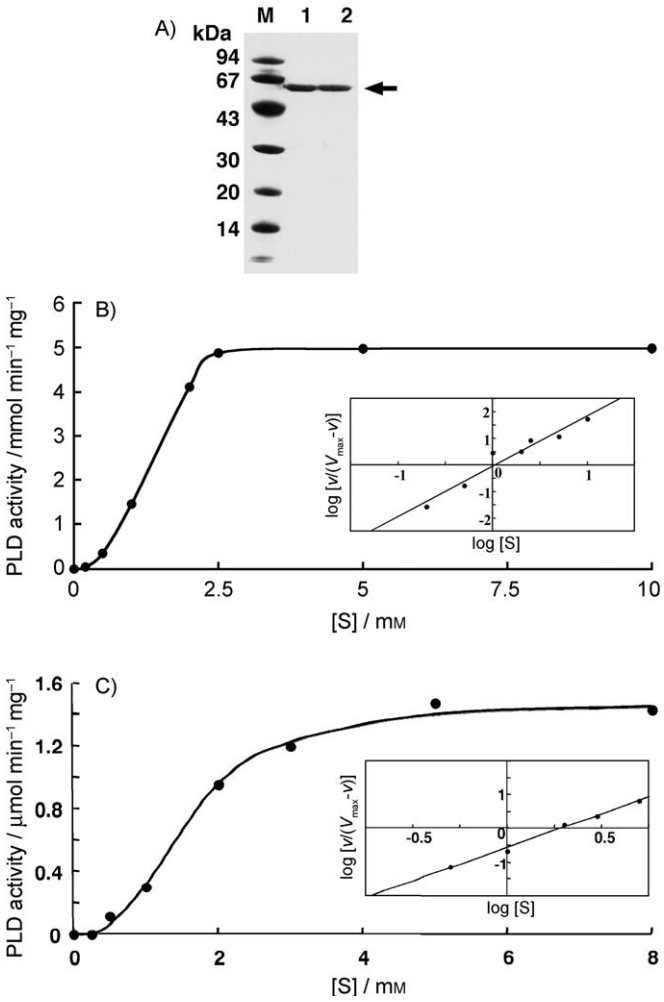


Figure 3. Kinetic analysis of the WT and the FRY mutant. A) SDS-PAGE analysis of the purified enzymes. The positions of the enzymes are indicated by an arrow. PLD activity versus substrate concentration plots of: B) the WT and C) FRY. Hill plots of the data are shown in small insets.

Table 1. Kinetic parameters of the WT and FRY in DOPC hydrolysis.

Enzyme	K_M [mM]	V_{max} [μmol min^{-1} per mg protein]	V_{max}/K_M	Hill coefficient
WT	1.5 ± 0.1	4700 ± 90	3.1×10^3	2.5 ± 0.3
FRY	3.0 ± 0.6	1.8 ± 0.2	0.6	1.6 ± 0.4

Figure 4 compares the transphosphatidylation activities of the WT and of FRY toward various cyclohexanols as acceptor compounds. Since transphosphatidylation competes with hydrolysis, the enzyme's ability to transfer the phosphatidyl group to the hydroxy group of the acceptor can be evaluated from the PX/PA values. Formation of the transphosphatidylated products (PX) was confirmed by identifying them with the aid of HPLC-MS (data not shown). When the reaction product of the 1,2,3-triol was subjected to periodate oxidation, the PX completely disappeared (Supporting Information). This suggests that an outer hydroxy group (1- or 3-OH), but not the center one (2-OH), of the 1,2,3-triol was linked to the phosphatidyl group.

In the cases of cyclohexanol and of cyclohexane-1,4-diol, the WT enzyme generated the corresponding PX more efficiently than the FRY. In contrast, FRY transferred the other acceptors—especially the 1,2-diols, triols, and *myo*-inositol—more efficiently than the WT. It seemed that the FRY mutant preferred cyclohexanols with two or more hydroxy groups that are close to each other, or ones with sterically bulkier (or larger) structures.

Discussion

The studies on protein engineering of *Streptomyces* PLDs reported so far have mainly focused on enhancing the stability or activity.[7] Hence, the current work is the first example of alteration of the substrate specificity of PLD. Until now, two plant PLDs had been reported to catalyze PI synthesis,[2] but the structure of the enzymatically synthesized PI had not been fully analyzed. Since industrial production of *Streptomyces* PLDs by fermentation has already been established by several companies, adding the PI-synthesizing activity to a *Streptomyces* PLD is of value for practical purposes.

The amino acid residues for the mutation (W187, Y191, and Y385) were chosen on the basis of the tertiary structure model of Sa-PLD in complexation with the substrate. These residues were oriented in the space that was expected to accommodate the head group or the acceptor compound in the substrate binding pocket. The reaction mechanism proposed in PMF-PLD,[5a] where H170 acts as the first nucleophile to attack the phosphorus atom, helped us to identify this space. Since the nucleophilic attack by H170 is an in-line S_N-2-type reaction, the nucleophile and the leaving group (i.e., head group) should be located on opposite sides of the phosphorus.

Of the three residues, Y385 seems to be a residue that limits the size of the head group of the substrate in PMF-PLD (Y390 in PMF-PLD).[5a] A computer-assisted substrate docking model in PMF-PLD also suggests that the head group contacts with that residue.[8] These suggestions are true for Sa-PLD because 15 PI-synthesizing mutant enzymes had amino acid replacement at position 385, and because one of the mutants had a replacement only at that position. However, no change was observed at position 385 in the other 10 mutants, suggesting that the other positions (187th and 191st) are also involved in the substrate recognition.

Out of the 25 positive mutants, only three had a single mutation at one of the three positions, while the others had two or three mutations. This result indicates that the mutation strategy we employed here, in which the three residues were mutated simultaneously, was effective for altering the enzyme's specificity. A similar mutational strategy for inverting the enantioselectivity of *Burkholderia cepacia* lipase was demonstrated by Nakano's research group.[9a] They constructed a combinatorial library by replacing four amino acid residues in the substrate binding pocket by use of a single-molecule PCR-linked in vitro expression (SIMPLEX) technique. They isolated

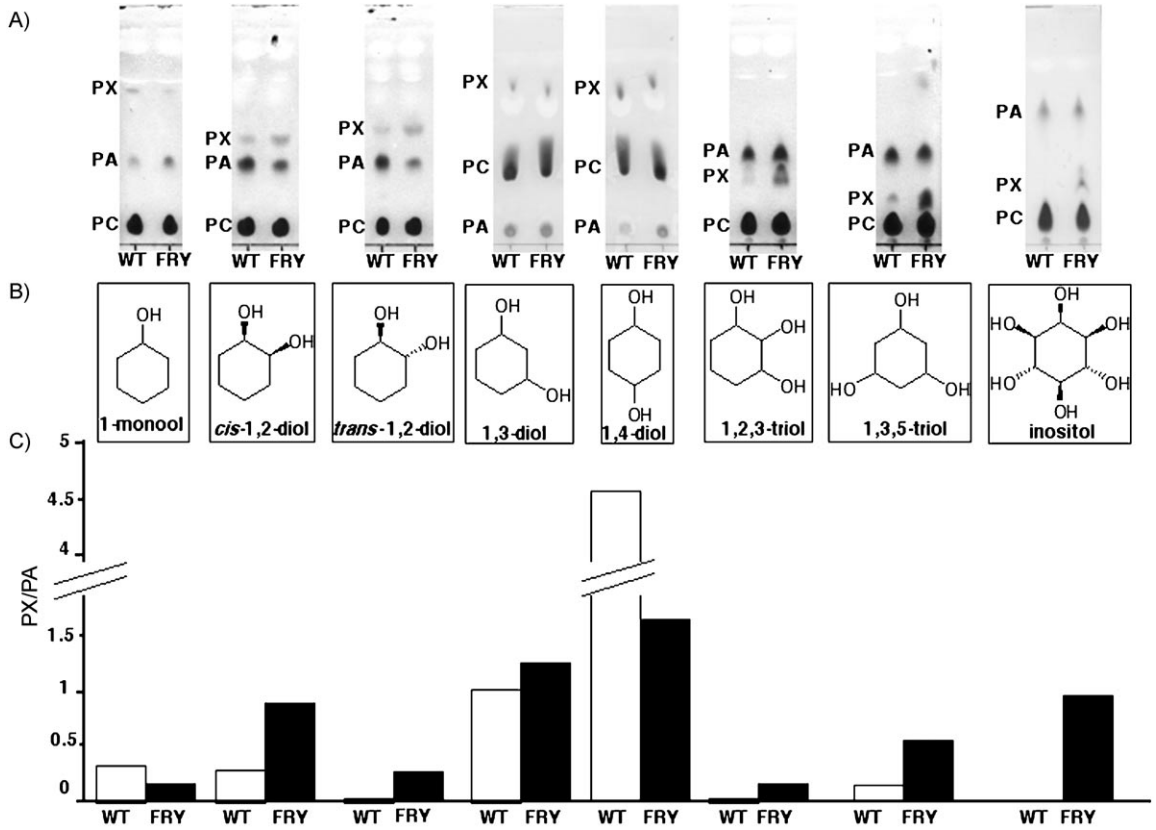


Figure 4. Comparison of the transphosphatidylation abilities of the WT and of FRY with various cyclohexanols as acceptors. A) TLC patterns of the reaction products. The positions of the phospholipids are shown as indicated. PX denote phosphatidylated products. B) The structures of the acceptors. Note that the 1,3-diol, 1,4-diol, 1,2,3-triol, and 1,3,5-triol used were mixtures of *cis–trans* isomers of unknown compositions. C) Transphosphatidylation abilities toward various cyclohexanols. The average PX/PA values obtained from three independent experiments are shown.

mutant lipases with inverted enantioselectivity towards *p*-nitrophenyl 3-phenylbutyrate. A more systematic and versatile strategy, namely a combinatorial active site test (CAST), was proposed by Reetz's research group.[10] They constructed small libraries by introducing mutations into two or three amino acids, the side chains of which resided next to the binding pocket, to isolate improved mutant enzymes such as a lipase,[10a,b] an epoxide hydrolase,[10c] and a cyclopentanone monooxygenase.[10d] Interestingly, Reetz's research group successfully further improved the enzymes by iterative CASTing or by combining mutations of different variants obtained from the initial CASTing. In our case, further improvement of PLD would be possible by repeating the mutation and screening with the mutant PLD (e.g., FRY) as the template.

Simultaneous mutation at three amino acids yields $20^3 = 8000$ variants, while theoretically at least 23964 colonies should be screened to achieve 95% coverage of this library size.[11] Moreover, if the degeneration of codons is taken into account, the number of colonies that should be screened in order to obtain reasonable coverage should be much larger. This means that our screening size of 10000 colonies was not sufficient to cover most variants in the library, implying that other positive clones might have been missed out. One approach to find such missed positive mutants would be the use of fuzzy neural network-assisted (FNN-assisted) screening.[9b] This strategy extracts hidden rules underlying sequences of variants and enzyme activity from the screening data with the aid of a bioinformatic algorithm, and it predicts better mutants that might have been missed out in the screening. Applying the FNN-assisted method for PLD engineering should facilitate the isolation of better mutant PLDs.

In our previous study, we speculated that H442 located on the C-terminal half domain of Sa-PLD functions as the nucleophile, from the result of a labeling experiment using an engineered PLD reconstituted from N-terminal and C-terminal halves.[12] However, this conclusion, including the tertiary structure of PLD-PMF, was refuted by several reports.[5] The tertiary structure of the intact Sa-PLD also does not support our previous conclusion. In addition, our present finding that altering the space located opposite to the H168 surely changes acceptor recognition implies that H168 is the nucleophile, and that our previous conclusion for the full-length Sa-PLD is incorrect.

The principle of the high-throughput screening method employed here is based on three factors: 1) PI forms an insoluble calcium salt, while PC does not, 2) the glycol group of the inositol ring is cleaved by periodate oxidation to form an aldehyde, and 3) the aldehyde group reacts with 4-hydrazino-7-nitrobenzofurazan (NBD-hydrazine, NBD-H) to form the strongly fluorescent NBD-hydrazone.[13] Out of the 48 clones isolated by the screening, only four were pseudopositive, according to the

TLC analysis. This indicates that the screening method worked well. The method should be useful for screening mutated PLDs with activities for synthesizing other artificial phospholipids with glycol-containing compounds such as oligosaccharides.

It is remarkable that the FRY mutant generated 1(3)-PI and 4(6)-PI, but not 2-PI and 5-PI. The formation of each PI isomer depends on how the enzyme recognizes the *myo*-inositol molecule. In the reaction mechanism of PMF-PLD, H448 acts as a general base that deprotonates the water or the acceptor. Therefore, the substrate binding pocket should accommodate the *myo*-inositol molecule in a suitable orientation so that H442 of Sa-PLD (corresponding to H448 of PMF-PLD) can deprotonate the particular hydroxy group of the ring. FRY might recognize the substrate orientation by forming hydrogen bonds between side chains and the hydroxy groups of the ring. *myo*-Inositol has one axial (2-OH) and five equatorial hydroxy groups (1-, 3-, 4-, 5-, and 6-OH) in its chair conformation. The axial 2-OH might therefore react with the phosphatidyl group with greater difficulty than the equatorial hydroxy groups, because, to do so, the inositol ring would have to be positioned in a very different orientation. FRY might not be able to accommodate the inositol in such an orientation to place the axial 2-OH close to H442, which could be the reason why 2-PI was not formed by FRY. The reason for 5-PI not being formed is not as clear as that for 2-PI, but interactions between the hydroxy groups (especially 2-OH) and some side chains in the binding pocket could be involved.

Among the cyclohexanols tested for transphosphatidylation, the 1,3- and 1,4-diols reacted better than the others in the presence of both WT and FRY. This superior reactivity of the 1,3- and 1,4-diols can be explained in terms of low degrees of steric hindrance due to the absence of vicinal hydroxy groups.

As for the 1,2-diols, the *cis*-1,2-diol has one equatorial and one axial hydroxy group, while the *trans*-1,2-diol has two equatorial groups.[14] The equatorial–axial conformation in *cis*-1,2-diol is the same as that for 1-OH and 2-OH (or 3-OH and 2-OH) in *myo*-inositol. In the FRY-mediated reaction, the *cis*-1,2-diol was transphosphatidylated better than the *trans*-1,2-diol (Figure 4). Moreover, in the PI synthesis, the amount of 1(3)-PI was approximately twice that of 4(6)-PI. These results again imply the involvement of the axial hydroxy group (e.g., 2-OH in *myo*-inositol) at the vicinal position of the phosphodiester-forming hydroxy group for the proper substrate orientation.

The transphosphatidylated product of the 1,2,3-triol was revealed to have the phosphatidyl group at an outer hydroxyl group (1-OH or 3-OH), but not at the center one. This is because the enzyme attacked the triol from the easily accessible (less intricate) side. This is in agreement with the case of the 1,3,5-triol, which reacted better than the 1,2,3-triol.

The kinetic analysis showed a significant difference between the WT and FRY in PC-hydrolyzing activity. The K_M value of FRY was 3.0 mM. While slightly higher, the K_M value is comparable to that of the WT. In contrast, the V_{max} value of FRY had dramatically decreased and the catalytic efficiency of the FRY mutant was approximately 5200 times lower than that of the WT. FRY has two amino acid substitutions, W187F and Y191R, in the substrate binding pocket of Sa-PLD.

Considering the involvement of the mutations W187F and Y191R in PC hydrolysis, the significant decrease in the catalytic efficiency might be explained in two possible ways. Firstly, the positive charge provided by the Y191R mutation might have hindered the stable binding of the positively charged quaternary ammonium group of the choline moiety through electrostatic interaction.

Secondly, the substitutions might affect stabilization by cation–π interactions between the aromatic residues W187 and/or Y191 and the quaternary ammonium group of the substrate. The cation–π interactions represent a genre of protein–ligand recognition motifs,[15] and a number of G protein-coupled receptors and neuroreceptors bind to ligands through these interactions.[15b–e] The contributions of this type of interactions to catalysis and binding for enzymes have also been investigated.[15f,g] In *Bacillus cereus* phospholipase C (PLC_{BC}), removal of the putative cation–π interaction resulted in a >200-fold decrease in k_{cat}; this suggests that an aromatic residue (F66 in PLC_{BC}) plays an important role in stabilizing the positive charge on the quaternary ammonium group of the substrate diC6PC through a putative cation–π interaction.[15f] Considering the size of the substrate binding pocket in Sa-PLD, it is possible for the choline moiety to enter into cation–π interactions with W187, Y191, and/or Y385. Since the aromatic ring of tryptophan is believed to enter into stronger cation–π interactions than tyrosine or phenylalanine,[15a] the mutation on W187 of Sa-PLD seems to affect the hydrolytic activity substantially.

In conclusion, we have achieved the creation of PI-synthesizing *Streptomyces* PLDs. However, the mutant PLD has two drawbacks. One is its very low catalytic efficiency (5200-fold decrease), requiring large amounts of enzymes to achieve a practical reaction rate, and the other is its low selectivity toward the position in *myo*-inositol, resulting in the generation of mixtures of several PI isomers. Further study will be necessary in order to isolate other mutants capable of synthesizing particular PI isomers specifically with reasonable catalytic efficiency.

Experimental Section

Chemicals: NBD-H was purchased from Fluka. *myo*-Inositol, cyclohexanol, and cyclohexane-1,4-diol (*cis* and *trans* mixture) were obtained from Wako (Osaka, Japan). *cis*-Cyclohexane-1,2-diol and *trans*-cyclohexane-1,2-diol were obtained from Aldrich. Cyclohexane-1,3-diol (*cis* and *trans* mixture), cyclohexane-1,3,5-triol (*cis* and *trans* mixture), and cyclohexane-1,2,3-triol (*cis* and *trans* mixture) were obtained from Tokyo Chemical Industries (Tokyo, Japan). DOPC was obtained from NOF Corporation (Tokyo, Japan). Soybean PI (99%) was obtained from Sigma. Soybean lecithin (SLP-PC70, containing approximately 70% PC), was provided by Tsuji Oil (Mie, Japan). Nitrocellulose membranes (Hybond-C) were obtained from GE Healthcare (Little Chalfont, UK).

Library construction strategy for introducing mutation: Mutations were introduced into the *pld* gene at the codons corresponding to W187, Y191, and Y385 by overlapping PCR with use of a pPELB-PLD-KS-II-CAT plasmid as the template (Supporting Information). Firstly, a 1748 bp DNA fragment, (fragment 1) including chloramphenicol acetyltransferase (*cat*) gene, *T7-lac* promoter, ribosome

binding site (RBS), *pelB* signal, and an N-terminal portion of the *pld* gene was amplified by PCR with primers PL-F1 (5′-CGTTGTAAAACGACGGCCAGTGA-3′) and OL-R1 (5′-GCCGTTGATCCCGCCCGTGATGGCCGTCT-3′). With the same template, another 617 bp fragment (fragment 2) was amplified with primers OL-F1 (5′-ATCACGGGCGGGATCAACGGCNNSAAGGACGACNNSCTCGACACCGCCCACCCG-3′) and OL-R2 (5′-GCCCCCGCTGCCGACGGCGCCGCGGTTGGCGGGATCGC-3′), introducing "NNS" mutations at the sites corresponding to W187 and Y191. Then, a 496 bp fragment (fragment 3) was amplified with primers OL-F2 (5′-GGCGCCGTCGGCAGCGGGGGCNNSTCCCAGATCAAGT-3′) and PL-R1 (5′-TAACCCTCACTAAAGGGAACAAA-3′), introducing an "NNS" mutation at the codon for Y385. Fragments 2 and 3 were connected by overlapping PCR by using the primers OL-F1 and PL-R1 to obtain a 1092 bp fragment (fragment 4). Finally, fragments 1 and 4 were connected with the primers PL-F1 and PL-R1 to yield a 2815 bp DNA fragment containing *cat*, *T7-lac* promoter, RBS, *pelB* signal, and the full-length *pld* with NNS mutations at the three codons.

The 2815 bp DNA fragments thus obtained were cut with SpeI and XhoI and ligated with SpeI and SalI-digested vector plasmid, pETKmS1-term. The ligation mixture was then introduced into *E. coli* DH5α host cells and streaked on LB-agar plates supplemented with chloramphenicol (30 μg mL^{-1}) and kanamycin (50 μg mL^{-1}). The *cat* gene included in the 2815 bp fragment enabled the selective growth of colonies possessing the recombinant plasmid with the inserted DNA fragment. Fresh LB liquid medium (1 mL per 90 mm diameter plate) was added to the colonies on the LB-agar plates. The cells were suspended in the liquid medium, and the cell suspension was recovered. From this cell suspension, the plasmids were prepared. The plasmid solution (mixture of PLD-expressing plasmids with various combination of mutations) thus obtained was used as the plasmid library.

High-throughput screening for PI-synthesizing mutant enzymes: A portion of the plasmid library was introduced into the expressing host, *E. coli* BL21 (DE3), and grown on LB-agar plates supplemented with kanamycin and chloramphenicol. Nitrocellulose membranes were put on the plates to transfer the colonies. While the LB plates were stored at 4 °C as the master plates, the membranes were placed on other agar plates containing a synthetic broth (a culture medium optimized for PLD production by the recombinants[16]) and incubated at 30 °C for 8 h. The membranes were then transferred onto other fresh agar plates of the synthetic broth containing isopropyl ß-D-thiogalactopyranoside (1 mM) and incubated further for 16 h at 30 °C to induce the expression of the pld gene. Since the recombinants were designed to excrete PLD out of the cells,[16] the synthesized PLD was expected to be adsorbed on the surface of the membrane at the positions of colonies.

After the induction, the membranes were washed briefly in sodium acetate/acetic acid buffer (50 mM, pH 5.6) containing Triton X-100 (0.5%) with the aid of a sonicating washing bath for 1 min to remove the cell debris. The membranes were then soaked into the substrate solution containing soybean lecithin (10%), *myo*-inositol (20%), and $CaCl_2$ (2%) and were incubated at 37 °C for 16 h for the enzyme reaction. The PI generated by the action of the mutated PLD was expected to form a water-insoluble calcium salt and to precipitate on the surface of the membrane at the position where the reaction occurred. After washing of the membranes with water to remove the remaining substrates, the membranes were soaked in $NaIO_4$ solution (10%) for 10 min for periodate oxidation, in which the inositol ring of PI was cleaved to form an aldehyde-containing lipid. The membranes were rinsed with water, and then an aqueous solution containing NBD-H (0.05%) and DMSO (10%) was spread onto the membranes (0.2 mL of the above solution was put on a 90 mm diameter membrane and spread by hand in a plastic sealing bag). NBD-H itself is a weakly fluorescent compound, but it shows strong fluorescence when it turns into a hydrazone after spontaneous coupling with an aldehyde.[13] The positive clones displaying PI-synthesizing activity were detected as strongly fluorescent spots on the membranes under UV light (365 nm), and the colonies corresponding to the positive spots were recovered from the master plates. The principle of the screening is shown in Scheme 1.

PLD-catalyzed PI synthesis: A mixture containing DOPC (1 mg, 1.27 μmol) dissolved in ethyl acetate (100 μL), *myo*-inositol (18 mg, 0.1 mmol) in sodium acetate buffer (50 mM, pH 5.6, 90 μL), and PLD solution (either the culture supernatant of the mutant *E. coli* or purified enzymes, 10 μL) was incubated at 37 °C with shaking for 16 h. HCl (1 M, 50 μL) and chloroform/methanol 2:1 (by vol., 200 μL) were then added in order to stop the reaction. After centrifugation, the lower layer containing the lipids was recovered. Approximately 5 μL of the resultant lipid solution was spotted on a silica gel TLC plate and developed with chloroform/petroleum ether/methanol/acetic acid (4:3:2:1, *v/v*). Phospholipids were visualized by spraying with Dittmer–Lester reagent.[17]

For the preparation of NMR samples, the PI-synthesis reaction was performed as follows. A mixture consisting of DOPC (180 mg, 0.23 mmol) dissolved in ethyl acetate (18 mL), *myo*-inositol (1080 mg, 6 mmol), the purified mutant PLD (2.5 mg), and sodium acetate buffer (50 mM, pH 5.6, 6 mL) was allowed to react at 37 °C for 12 h with stirring. The reaction was stopped by addition of HCl (1 M, 18 mL). The lipid was extracted with chloroform/methanol (2:1, *v/v*, 72 mL) and dried with anhydrous sodium sulfate; the solvent was then evaporated. The residual lipids were chromatographed on a silica gel column (Wakogel C-300) with use of chloroform/petroleum ether/methanol/acetic acid (4:3:2:1, *v/v*). When necessary, the inositol ring of PI was acetylated by treatment of the lipid (2 mg, 2.3 μmol) with pyridine/acetic anhydride (2:1, *v/v*, 1 mL) containing 4,4-dimethylaminopyridine (0.1 mg, 0.8 μmol) at room temperature for 14 h, followed by purification on a small silica gel column.

High-performance liquid chromatography–mass spectrometry (HPLC-MS) analysis of the phospholipids: HPLC-MS analysis of the phospholipids was performed with an HPLC system (Prominence, Shimadzu, Kyoto, Japan) fitted with a mass spectrometer (Model LCMS-2000EV, Shimadzu) and an atmospheric pressure chemical ionization (APCI) probe. The mass spectrometer was operated in the negative ion scanning mode with a scanning range of *m/z* 600–1100.

A silica gel column (4.6×150 mm, 3 μm particle size, Phenomenex, Torrance, CA) was used at a flow rate of 0.5 mL min^{-1}. A binary gradient system was employed as described by Rombaut et al.[18] with slight modification, with use of solvent A [chloroform/methanol/0.97 M formic acid–triethylamine buffer (pH 3.0) 87.5:12:0.5, *v/v*] and solvent B [chloroform/methanol/0.97 M formic acid–triethylamine buffer (pH 3.0) 28:60:12, *v/v*]. The elution program was a linear gradient with 0% solvent B at 0 min to 40% solvent B at 16 min. The mobile phase was brought back to the initial conditions after 17 min, and the column was allowed to elute with 100% solvent A until 21 min. Standard PI isomers were chemically synthesized from DOPA and appropriately protected *myo*-inositols, as described,[19] and used for peak identification (Supporting Information).

Enzymatic synthesis of phosphatidylcyclohexanols: A mixture consisting of DOPC (1 mg, 1.27 μmol) dissolved in dichloromethane (100 μL), cyclohexanols (cyclohexanol, *cis*-cyclohexane-1,2-diol, *trans*-cyclohexanediol, cyclohexane-1,3-diol, cyclohexane-1,4-diol, cyclohexane-1,2,3-triol, cyclohexane-1,3,5-triol, and *myo*-inositol, 100 μmol), and either the WT-PLD (1.25 ng) or a mutant PLD (1 μg) dissolved in sodium acetate buffer (50 mM, pH 5.6, 100 μL) was incubated with mixing at 30 °C for 18 h. The lipids were extracted and analyzed by TLC. The developing solvents were chloroform/acetone/methanol/acetic acid/water 65:20:10:10:3 (for cyclohexanol, *cis*-cyclohexane-1,2-diol, *trans*-cyclohexanediol, cyclohexane-1,2,3-triol, and cyclohexane-1,3,5-triol), chloroform/methanol/25% ammonia 65:35:5 (for cyclohexane-1,3-diol and cyclohexane-1,4-diol), and chloroform/petroleum ether/methanol/acetic acid 4:3:2:1 (for *myo*-inositol). Phospholipids were visualized with Dittmer–Lester reagent, and phospholipid compositions were calculated with the aid of NIH image software.

Other methods: Mutant PLDs were prepared from the culture supernatants of recombinant strains of *E. coli* (and purified when necessary) as described previously.[16] The hydrolytic activity of PLD was assayed with DOPC as the substrate.[16] The kinetic parameters of PLD were measured with the final substrate concentration ranging from 0.25 to 10 mM. Data were determined from at least three independent experiments.

^{1}H NMR spectra were recorded on a Bruker AMX 600 (600 MHz) spectrometer. NMR samples were dissolved in $CDCl_3$ or CD_3OD, and chemical shifts were reported in ppm relative to tetramethylsilane ($\delta = 0.00$ ppm) in $CDCl_3$ or in ppm relative to the residual undeuterated solvent (CD_3OD as $\delta = 3.35$ ppm). The spectra were measured at 27 °C unless otherwise noted.

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