Enzymatic Phosphatidylation of Thiamin, Pantothenic Acid, and Their Derivatives

Naomi Hidaka1, Masaaki Takami2 and Yukio Suzuki1,*

1Research Institute for Bioresources, Okayama University, 2–20–1 Chuo, Kurashiki 710–0046, Japan
2Central Research Laboratories, Kuraray Co., Ltd., 2045–1 Sakazu, Kurashiki 710–0801, Japan
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Summary Phospholipase D from Streptomyces sp. was found to catalyze the transfer reaction of the dipalmitoylphosphatidyl residue from 1,2-dipalmitoyl-3-sn-phosphatidylcholine to thiamin, pantothenic acid, and their derivatives in a biphasic system. The following phosphatidylated compounds were synthesized: 1,2-dipalmitoyl-3-sn-phosphatidylthiamin, 1,2-dipalmitoyl-3-sn-phosphatidylthiamin propyl disulfide, 1,2-dipalmitoyl-3-sn-phosphatidylthiamin tetrahydrofurfuryl disulfide, 1,2-dipalmitoyl-3-sn-phosphatidylpantothenic acid, and 1,2-dipalmitoyl-3-sn-phosphatidyl-pantothenyl ethyl ether.

Key Words transphosphatidylation reaction, phospholipase D, phosphatidythiamin, phosphatidylthiamin disulfides, phosphatidylpantothenic acid

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Phospholipase D (PLD) (EC 3.1.4.4) catalyzes the hydrolysis of the terminal phosphoester bond of glycerophospholipids and can bring about phosphatidylation. The transfer reaction of the phosphatidyl residue from phosphatidylcholine to primary lower alkanols has been seen using PLD first from cabbage leaves (1), and thereafter from microorganisms. A large variety of acceptors for transphosphatidylation have been reported. Among them, ethanol (1), ethanolamine (2), serine (3), and glycerol (4) are excellent acceptors, and L-ascorbic acid (5), nucleosides (6, 7), primary higher alkanols with more than 6-carbon chains (8), saccharides and sugar alcohols (9) are also good acceptors. To improve the characteristics of water-soluble physiologically active compounds, phosphatidylation may be a suitable method, because the phosphatidyl residue is well characterized as a non-toxic carrier moiety that has a high affinity for cell membranes and great hydrophobicity to prevent degradation. In 1992–1993, we reported that PLD from Streptomyces sp. catalyzed the transfer reaction of the dipalmitoylphosphatidyl residue from 1,2-dipalmitoyl-3-sn-phosphatidylcholine (DPPC) to pyridoxine and riboflavin, respectively, in a biphasic system using a mixture of CHCl3 and acetate buffer, to afford 5’-(1,2-dipalmitoyl-3-sn-phosphatidyl)-pyridoxine (DPP-PN) and 5’-(1,2-dipalmitoyl-3-sn-phosphatidyl)-riboflavin (DPP-B2) (10–13). Moreover, we synthesized phosphatidylated derivatives of kojic acid, arbutin, genipin, dihydroxyacetone, and various aromatic hydroxy compounds via transphosphatidylation reaction by PLD (14–19). This paper deals with the enzymatic synthesis of phosphatidylated compounds of thiamin, pantothenic acid, and their derivatives (20, 21).

Materials and Methods

Enzymes and chemical reagents. PLD from Streptomyces sp., 1,2-dipalmitoyl-3-sn-phosphatidic acid sodium salt (DPPA-Na), and 1,2-dipalmitoyl-3-sn-phosphatidylethanol (DPP-Et) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). 1,2-Dipalmitoyl-3-sn-phosphatidylcholine (DPPC), thiamin hydrochloride, and sodium pantothenic acid were obtained from Nippon Fine Chemical Co., Ltd. (Osaka, Japan). Nacalai Tesque, Inc. (Kyoto, Japan), and Tokyo Kasei Industrial Co., Ltd. (Tokyo, Japan), respectively. Thiamin propyl disulfide (TPD), thiamin allyl disulfide (TAD), and thiamin tetrahydrofurfuryl disulfide (TTFD) were kindly supplied by Takeda Chemical Industries Ltd. (Osaka, Japan). Pantothenyl ethyl ether (PaOEt), acetylpantothenyl ethyl ether (AcPaOEt), and benzoylpantothenyl ethyl ether (BzPaOEt) were also kindly supplied by Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). Other reagents used were of analytical grade from commercial sources.

Analyses of reaction mixture with thin-layer chromatography (TLC). To a 50-μL reaction mixture, 50 μL of 1 N HCl was added and well mixed, and then the mixture was extracted with 100 μL of CHCl3-MeOH (3 : 1, v/v). The organic layer was put on a TLC-plate. TLC was performed with a silica gel 60F254 plate (E. Merck Co., Darmstadt, Germany) and CHCl3-MeOH-H2O (3 : 1 : 0.1, or 3 : 2 : 0.2, v/v/v) as the solvent. Phosphatidylated compounds were detected by spraying with 5% phosphomolybdic acid in ethanol, followed by heating, and further analyzed by a Shimadzu Flying-Spot Scanner CS-9000 (absorbance at 500 nm). The contents of phosphatidylated compounds were calculated according to the area observed on the scanner.
**Enzymatic synthesis of phosphatidylthiamin.** DPPC (1.25 g, 1.7 mmol) and thiamin hydrochloride (3.4 g, 10 mmol) were mixed with 12.5 mL of CHCl₃ and 12.5 mL of 0.1 M sodium acetate buffer (pH 5.6) containing 350 units of PLD, and the mixture was incubated at 37°C for 24 h with vigorous stirring. To the reaction mixture, 12.5 mL of 1 N HCl was added to stop the enzymatic reaction under vigorous stirring, and the mixture was extracted with 25 mL of CHCl₃. The CHCl₃ layer was washed two times using water saturated with NaCl, dried over anhydrous sodium sulfate overnight, concentrated in vacuo, and chromatographed on a silica gel (Wakogel C-200, Wako Pure Chemical Industries, Ltd., Osaka, Japan) column. From the eluate with CHCl₃-MeOH (3 : 1, v/v), the phosphatidylated derivative of thiamin was obtained and purified from ethanol as white powder (410 mg, 27% yield based on DPPC), and its structure was identified as 1,2-dipalmitoyl-3-phosphatidylthiamin (DPPC-P) by comparing the following spectral data with those of thiamin: F AB mass, m/z 896; 1H-NMR (δ ppm) 11.0 (1H, s, thiazole 2-CH), 8.14 (1H, s, pyrimidine 6-CH), 5.68 (2H, s, bridge CH₂), 5.22 (1H, m, glycerol CHO), 4.40 (1H, m, glycerol CH₂OP), 3.47 (1H, m, glycerol CH₂OP), 4.10 (2H, m, glycerol CH₂O), 3.97 (2H, t, thiazole β-CH₂ of side chain), 3.11 (2H, t, thiazole α-CH₂ of side chain), 2.52 (3H, s, pyrimidine 2'-CH₃), 2.47 (3H, s, thiazole 4-CH₃), 2.28 (4H, m, palmitoyl CH₂), 1.58 (4H, m, palmitoyl CH₂), 1.25 (48H, m, palmitoyl CH₂), 0.89 (6H, t, palm- toyl CH₂); 13C-NMR (δ ppm) 173.5 (palmitoyl C-O), 173.1 (palmitoyl C-O), 169.5 (pyrimidine C-4'), 162.3 (pyrimidine C-2'), 157.7 (thiazole C-2), 157.5 (pyrimidine C-6'), 142.4 (thiazole C-4), 135.2 (thiazole C-5), 104.6 (pyrimidine C-5'), 70.3 (glycerol CHO), 63.5 (thiazole β-CH₂ of side chain), 63.1 (glycerol CH₂OP), 62.7 (glycerol CH₂O), 52.3 (bridge CH₂), 34.3–22.7 (palmitoyl CH₂), 28.5 (thiazole α-CH₂ of side chain), 25.6 (pyrimidine 2'-CH₃), 14.1 (palmitoyl CH₂), 12.5 (thiazole 4-CH₃). NMR spectral data (in D₂O of thiamin (22, 23): 1H-NMR (δ ppm) 9.83 (1H, s, thiazole 2-CH), 8.13 (1H, s, pyrimidine 6-CH), 5.68 (2H, s, bridge CH₂), 3.96 (2H, t, thiazole β-CH₂ of side chain), 2.36 (2H, t, thiazole α-CH₂ of side chain), 2.70 (3H, s, pyrimidine 2'-CH₃), 2.62 (3H, s, thiazole 4-CH₃); 13C-NMR (δ ppm) 164.2 (pyrimidine C-4'), 163.8 (pyrimidine C-2'), 155.3 (thiazole 2-CH₂), 146.4 (pyrimidine C-6'), 143.6 (thiazole C-4), 137.3 (thiazole C-5), 106.5 (pyrimidine C-5'), 61.0 (thiazole β-CH₂ of side chain), 51.0 (bridge CH₂), 30.3 (thiazole α-CH₂ of side chain), 21.9 (pyrimidine 2'-CH₃), 12.2 (thiazole 4-CH₃).

**Enzymatic synthesis of phosphatidylthiamin propyl disulfide.** DPPC (0.206 g, 0.28 mmol) and thiamin propyl disulfide (TPD) (1.0 g, 2.81 mmol) were mixed with 20 mL of ethyl acetate and 20 mL of 0.1 M sodium acetate buffer (pH 5.6) containing 80 units of PLD, and the mixture was incubated at room temperature for 16 h with vigorous stirring. To the reaction mixture, 40 mL of 1 N HCl was added and well mixed. The mixture was extracted with 80 mL of CHCl₃-MeOH (3 : 1, v/v) and then the desired band was scraped out and extracted three times with CHCl₃-MeOH (1 : 1, v/v). The extract was concentrated in vacuo, to afford white powder of 1,2-dipalmitoyl-3-phosphatidylthiamin propyl disulfide (DPP-TPD) (40 mg, 15% yield based on DPPC). Its structure was identified by the following spectral data: FAB-mass, m/z 987; 1H-NMR (δ ppm) 8.05 (1H, s, pyrimidine 6'-CH), 5.23 (1H, m, glycerol CHO), 4.75 (2H, s, bridge CH₂), 4.40 (1H, m, glycerol CH₂OP), 3.47 (1H, m, glycerol CH₂OP), 4.14 (2H, m, glycerol CH₂O), 3.95 (2H, t, propyl 1-CH₃), 2.85 (2H, t, ethyl β-CH₂), 2.47 (3H, s, pyrimidine 2'-CH₃), 2.43 (2H, t, ethyl α-CH₂), 2.27 (4H, m, palmitoyl CH₂), 2.06 (3H, s, vinyleryl CH₂), 1.57 (4H, m, palmitoyl CH₂), 1.56 (2H, m, propyl 2-CH₂), 1.27 (48H, m, palmitoyl CH₂), 0.95 (3H, t, propyl CH₃), 0.89 (6H, t, palmitoyl CH₃); 13C-NMR (δ ppm) 173.3 (palmitoyl C-O), 172.9 (palmitoyl C-O), 164.0 (pyrimidine C-4'), 163.4 (amide C-O), 161.4 (pyrimidine C-2'), 145.1 (pyrimidine C-6'), 136.8 (vinyleryl 1/2 C), 132.7 (vinyleryl 1/2 C), 109.0 (pyrimidine C-5'), 70.0 (glycerol CHO), 63.0 (glycerol CH₂OP), 62.2 (glycerol CH₂O), 61.9 (ethyl β-CH₂), 40.4 (bridge CH₂), 38.2 (ethyl α-CH₂), 33.4–22.0 (palmitoyl CH₂), 31.2 (propyl 1-CH₂), 21.4 (pyrimidine 2'-CH₃), 20.9 (propyl 2-CH₂), 18.2 (vinyleryl CH₂), 13.1 (palmitoyl CH₂), 12.0 (propyl CH₂), NMR spectral data (in CDCl₃ of TPD: 1H-NMR (δ ppm): 8.07 (1H, s, pyrimidine 6'-CH), 4.42 (2H, s, bridge CH₂), 3.73 (2H, t, propyl 1-CH₂), 2.85 (2H, t, ethyl β-CH₂), 2.63 (3H, s, pyrimidine 2'-CH₃), 2.54 (2H, t, ethyl α-CH₂), 2.16 (3H, s, vinyleryl CH₂), 1.56 (2H, m, propyl 2-CH₂), 0.96 (3H, t, propyl CH₃); 13C-NMR (δ ppm) 164.0 (pyrimidine C-4'), 163.9 (amide C-O), 161.0 (pyrimidine C-2'), 143.2 (pyrimidine C-6'), 137.5 (vinyleryl 1/2 C), 131.7 (vinyleryl 1/2 C), 109.4 (pyrimidine C-5'), 58.9 (ethyl β-CH₂), 40.9 (bridge CH₂), 38.6 (ethyl α-CH₂), 32.0 (propyl 1-CH₂), 21.5 (pyrimidine 2'-CH₃), 20.8 (propyl 2-CH₂), 18.3 (vinyleryl CH₂), 12.3 (propyl CH₁).

**Enzymatic synthesis of phosphatidylthiamin tetrahydrofurfuryl disulfide.** DPPC (0.8 g, 1.09 mmol) and thiamin tetrahydrofurfuryl disulfide (TTFD) (4.0 g, 10.0 mmol) were mixed with 40 mL of ethyl acetate and 40 mL of 0.1 M sodium acetate buffer (pH 5.6) containing 80 units of PLD, and the mixture was incubated at room temperature for 16 h with vigorous stirring. To the reaction mixture, 40 mL of 1 N HCl was added and well mixed. The mixture was extracted with 80 mL of CHCl₃-MeOH (3 : 1, v/v). The organic layer was washed two times using water saturated with NaCl, dried over anhydrous sodium sulfate overnight, and concentrated. The concentrate was developed on a preparative silica gel TLC plate with CHCl₃-MeOH (3 : 1, v/v), and then the desired band was scraped out and extracted three times with CHCl₃-MeOH (1 : 1, v/v), and the extract was concentrated in vacuo, and chromatographed on a silica gel (Wakogel C-200) column with CHCl₃-MeOH (3 : 1, v/v), and then the desired band was scraped out and extracted three times with CHCl₃-MeOH (1 : 1, v/v).
vacuo, to afford 1,2-dipalmitoyl-3-sn-phosphatidylthiamin tetrahydrofurfuryl disulfide (DPP-TTFD) (240 mg, 21% yield based on DPPC). Its structure was confirmed on the basis of the following spectral data: 1H-NMR (δ ppm), 8.00 (1H, s, pyrimidine C-6'), 5.22 (1H, m, glycerol CHO), 4.79 (2H, s, bridge CH2), 4.39 (1H, m, glycerol CH2OP), 4.37 (1H, m, glycerol CH2OP), 4.15 (2H, m, glycerol CH2O), 3.92 (2H, d, S-bridge CH2), 3.82 (2H, t, tetrahydrofurfuryl 4-CH2), 3.70 (1H, t, tetrahydrofurfuryl 1-CH), 2.85 (2H, t, ethyl β-CH2), 2.59 (2H, t, ethyl α-CH2), 2.44 (3H, s, pyrimidine 2'-CH3), 2.28 (4H, m, palmitoyl CH2), 2.05 (3H, s, vinylenyl CH3), 1.98 (2H, m, tetrahydrofurfuryl 3-CH2), 1.87 (2H, m, tetrahydrofurfuryl 2-CH2), 1.54 (4H, m, palmitoyl CH2), 1.24 (48H, m, palmitoyl CH2), 0.87 (6H, t, palmitoyl CH3); 13C-NMR (δ ppm), 173.7 (palmitoyl C-0), 173.5 (palmitoyl C=O), 166.8 (pyrimidine C-4'), 163.8 (amide C-O), 156.0 (pyrimidine C-2'), 153.2 (pyrimidine C-6'), 136.5 (vinylene 1/2 C), 133.6 (vinylene 1/ 2 C), 108.7 (pyrimidine C-5'), 70.9 (glycerol CHO), 70.8 (ethyl β-CH2), 68.4 (tetrahydrofurfuryl 4-CH2), 63.7 (glycerol CH2OP), 63.0 (glycerol CH2O), 44.9 (bridge CH2), 40.2 (ethyl α-CH2), 34.3–22.9 (palmitoyl CH2), 31.1 (tetrahydrofurfuryl 1-CH), 30.9 (tetrahydrofurfuryl 3-CH2), 29.6 (S-bridge CH2), 25.2 (tetrahydrofurfuryl 2-CH2), 25.0 (pyrimidine 2'-CH3), 19.7 (vinylene CH3), 14.3 (palmitoyl CH3). NMR spectra data (in D2O): 1H-NMR (δ ppm): 8.00 (1H, s, pyrimidine C-6'), 4.80 (2H, s, bridge CH2), 3.92 (2H, d, S-bridge CH2), 3.74 (2H, t, tetrahydrofurfuryl 4-CH2), 3.73 (1H, t, tetrahydrofurfuryl 1-CH), 2.85 (2H, t, ethyl β-CH2), 2.61 (2H, t, ethyl α-CH2), 2.44 (3H, s, pyrimidine 2'-CH3), 2.05 (3H, s, vinylenyl CH3), 2.01 (2H, m, tetrahydrofurfuryl 3-CH2), 1.89 (2H, m, tetrahydrofurfuryl 2-CH2); 13C-NMR (δ ppm): 168.0 (pyrimidine C-4'), 163.7 (amide C-O), 162.3 (pyrimidine C-2'), 156.4 (pyrimidine C-6'), 136.5 (vinylene 1/2 C), 132.2 (vinylene 1/ 2 C), 108.3 (pyrimidine C-5'), 68.3 (tetrahydrofurfuryl 4-CH2), 60.2 (ethyl β-CH2), 45.2 (bridge CH2), 40.3 (ethyl α-CH2), 32.8 (tetrahydrofurfuryl 1-CH), 30.8 (S-bridge CH2), 30.8 (tetrahydrofurfuryl 3-CH2), 25.8 (tetrahydrofurfuryl 2-CH2), 25.6 (pyrimidine 2'-CH3), 19.3 (vinylene CH3).

Enzymatic synthesis of phosphatidylpantothenic acid. DPPC (1.0 g, 1.36 mmol) and pantothenic acid sodium salt (PaA-Na) (3.3 g, 13.6 mmol) were mixed with 30 mL of ethyl acetate and 30 mL of 0.2 M sodium acetate buffer (pH 5.6) containing 100 units of PLD, and the mixture was incubated at room temperature for 6 h with vigorous stirring. To the reaction mixture, 50 mL of 1 n HCl was added and well mixed. The mixture was extracted with 100 mL of CHCl3-MeOH (3:1, v/v). The organic layer was washed two times with water saturated with NaCl, dried over anhydrous sodium sulfate overnight, and concentrated. The concentrate was chromatographed on a silica gel (Wakogel C-200) column with CHCl3-MeOH (8:1, v/v). The desired fractions were checked using TLC, pooled, concentrated, and purified from ethanol to afford a white powder of 1,2-dipalmitoyl-3-sn-phosphatidylpantethenyl ethyl ether (DPP-PaOEt) (300 mg, 25% yield based on DPPC). Its structure was confirmed on the basis of the following spectral data: 1H-NMR (δ ppm), 5.22 (1H, m, glycerol CHO), 4.39 (1H, m, glycerol CH2OP), 4.16 (2H, m, glycerol CH2O), 3.96 (1H, s, pantoyl CH), 3.47 (2H, m, pantoyl CH2), 3.44 (2H, t, N-propyl CH2O), 3.39 (2H, m, N-propyl NCH3), 3.34 (2H, m, N-propyl CH2), 2.29 (4H, m, palmitoyl CH2), 1.78 (2H, m, ethyl CH2), 1.57 (4H, m, palmitoyl CH2), 1.25 (48H, m, palmitoyl CH2), 1.20 (3H, t, ethyl CH3), 0.90 (3H, t, palmitoyl CH3), 0.88 (6H, t, palmitoyl CH3), 0.87 (3H, s, pantoyl CH3); 13C-NMR (δ ppm), 174.4 (palmitoyl C=O), 173.8 (palmitoyl C=O), 173.6 (palmitoyl C=O), 73.5 (pantoyl CH), 71.8 (pantoyl CH2), 70.8 (N-propyl CH2O), 69.0 (glycerol CHO), 66.5 (ethyl CH2), 63.9 (glycerol CH2O). 39.3
(pantoyl C), 37.4 (N-propyl NCH2), 34.5–22.9 (palmitoyl CH2), 29.4 (N-propyl CH2), 21.3 (pantoyl CH3), 20.7 (pantoyl CH3), 15.3 (ethyl CH3), 14.3 (palmitoyl CH3). NMR spectral data (in CDCl3) of PaOEt: 1H-NMR (ppm): 3.97 (1H, s, pantoyl CH), 3.49 (2H, m, pantoyl CH2), 3.44 (2H, t, N-propyl CH2O), 3.39 (2H, m, N-propyl NCH2), 1.79 (2H, m, ethyl CH2), 1.21 (3H, t, ethyl CH3), 0.96 (3H, s, pantoyl CH3), 0.91 (3H, s, pantoyl CH3); 13C-NMR (δ ppm): 173.9 (pantoyl C=O), 77.3 (pantoyl CH), 70.9 (N-propyl CH2O), 68.9 (pantoyl CH2), 66.4 (ethyl CH2), 39.2 (pantoyl C), 37.3 (N-propyl NCH2), 29.3 (N-propyl CH3), 21.1 (pantoyl CH3), 20.4 (pantoyl CH3), 15.1 (ethyl CH3). The structures of these five phosphatidylated compounds are shown in Fig. 1.

**Instrumental analyses.** FAB mass spectra (pos.) were measured on a JEOL JMS-HX 100 mass spectrometer. 1H- and 13C-NMR spectra were recorded with a JEOL GSX-270 spectrometer with tetramethylsilane as an internal standard in CDCl3.

**Results.**

1. **Transphosphatidylation reaction of DPPC to thiamin and its derivatives by PLD.**

We examined the transfer reaction of the dipalmitoylphosphatidyl residue from DPPC to thiamin with PLD in a biphasic system using a mixture of ethyl acetate and acetate buffer. The reaction mixture containing DPPC (10 mg, 13.6 μmol) in 1 mL of ethyl acetate, thiamin HCl (46 mg, 136 μmol) in 1 mL of 0.2 M sodium acetate buffer (pH 5.6) (adjusted to pH 5.6 with NaOH), and PLD (3 units) in 10 μL of 0.2 M sodium acetate buffer (pH 5.6) was added, and then incubated at room temperature for 3 h under stirring. After incubation, the reaction mixture was analyzed by TLC as described in "Materials and Methods." a. DPPC+B1+PLD; b. DPPC+TPD+PLD; c. DPPC+PaA+PLD. TLC was done with a solvent system of CHCl3-MeOH-H2O (a. c. 3 : 2 : 0.2; b. 3 : 1 : 0.1, v/v/v). 1. DPPC; 2. B1 (or TPD, PaA); 3. organic layer; 4. water layer.

**Fig. 2.** Thin-layer chromatograms of transphosphatidylated reaction products obtained from DPPC and thiamin (thiamin propyl disulfide or pantothenic acid) by PLD. DPPC (10 mg, 13.6 μmol) and thiamin HCl (or the following vitamins) (136 μmol) were added to a mixture of 1 mL of ethyl acetate and 1 mL of 0.2 M sodium acetate buffer (pH 5.6) (adjusted to pH 5.6 with NaOH), and PLD (3 units) in 10 μL of 0.2 M sodium acetate buffer (pH 5.6) was added, and then incubated at room temperature for 3 h under stirring. After incubation, the reaction mixture was analyzed by TLC as described in “Materials and Methods.” a. DPPC+B1+PLD; b. DPPC+TPD+PLD; c. DPPC+PaA+PLD. TLC was done with a solvent system of CHCl3-MeOH-H2O (a. c. 3 : 2 : 0.2; b. 3 : 1 : 0.1, v/v/v). 1. DPPC; 2. B1 (or TPD, PaA); 3. organic layer; 4. water layer.

**Fig. 3.** Thin-layer chromatograms of transphosphatidylated reaction products obtained from DPPC and the derivatives of thiamin and pantothenic acid. DPPC and thiamin derivatives (or pantothenic acid derivatives) were incubated with PLD at room temperature for 3 h under the same experimental conditions as described in Fig. 2. TLC was done with CHCl3-MeOH-H2O (3 : 1 : 0.1, v/v/v). 1. DPPC+TAD+PLD; 2. DPPC+TTFD+PLD; 3. DPPC+PaOEt+PLD; 4. DPPC+AcPaOEt+PLD; 5. DPPC+BzPaOEt+PLD.
Transphosphatidylation Reaction to Vitamins by Phospholipase D

DPPC to several thiamin derivatives, such as TAD, TPD, and TTFD, by PLD were examined under the same experimental conditions. In these reactions, a new spot (X2) having a lower Rf value than that of TPD was observed on TLC analysis (Fig. 2). TTFD also gave a new spot (X3) having a lower mobility than that of TTFD on the thin-layer chromatogram developed with CHCl3-MeOH-H2O (3 : 1 : 0.1, v/v/v), but TAD did not (Fig. 3).

2. Transphosphatidylation reaction of DPPC to pantothenic acid and its derivatives by PLD

When DPPC (13.6 µmol) and pantothenic acid sodium salt (136 µmol) were incubated in the mixture of 1 mL of ethyl acetate and 1 mL of 0.2 M sodium acetate buffer (pH 5.6) containing 3 units of PLD at room temperature for 3 h under stirring, a new minor spot (X4) having an Rf value different from those of DPPC and DPPA-Na was observed with the organic layer on TLC analysis (Fig. 2). In similar transphosphatidylation reactions of DPPC to several pantothenic acid derivatives, such as PaOEt, AcPaOEt, and BzPaOEt, by PLD, PaOEt and AcPaOEt gave a new spot (X5) and a new trace spot (X6) having lower mobilities than those of PaOEt and AcPaOEt, respectively, on thin-layer chromatograms developed with CHCl3-MeOH-H2O (3 : 1 : 0.1, v/v/v), but BzPaOEt did not (Fig. 3).

The above mentioned six new spots were not observed on TLC analysis when the reaction mixture with boiled PLD or without PLD (or DPPC, thiamin, TPD, TTFD, PaA, PaOEt, AcPaOEt) was incubated. These results suggest that these new spots corresponded to the transphosphatidylated products of thiamin, TPD, TTFD, PaA, PaOEt, and AcPaOEt, respectively. These five products corresponding to the five spots (X1, X2, X3, X4, and X5) were isolated from the reaction mixtures in a semipreparative scale by silica gel column chromatography or preparative silica gel TLC, and their structures were confirmed as DPP-B1, DPP-TPD, DPP-TTFD, DPP-PaA, and DPP-PaOEt on the basis of the spectral evidence described in “Materials and Methods.”

3. Time-course of transphosphatidylation reaction of DPPC to thiamin and thiamin propyl disulfide by PLD

The time-course of the transphosphatidylation reaction of DPPC to thiamin by PLD is shown in Fig. 4. The formation of DPP-B1—the transfer reaction of dipalmitylphosphatidyl residue from DPPC to thiamin in a biphasic system—occurred in the early stage of the reaction. After a 6 h-incubation, most of the DPPC was consumed and DPP-B1 attained a maximum accumulation (over 90% yield based on DPPC), but a slight decrease of DPP-B1 and the formation of DPPA-Na were observed after a 24 h-incubation. The rapid formation of DPP-TPD also occurred efficiently in the early stage of the reaction (about 65% yield based on DPPC, after a 6 h-incubation), while a gradual increase of DPPA-Na was observed (about 20% yield based on DPPC) (Fig. 5).

Discussion

In this paper, five transphosphatidylated compounds of water-soluble vitamins and their derivatives, DPP-B1, DPP-TPD, DPP-TTFD, DPP-PaA, and DPP-PaOEt, were synthesized by the transfer reactions of dipalmitylphosphatidyl residue from DPPC to thiamin, pantothenic acid, and their derivatives, respectively, by PLD from Streptomyces sp. in a biphasic system using a mixture of ethyl acetate and acetate buffer (Fig. 1). We first examined the transphosphatidylation reaction of DPPC to thiamin with PLD in a biphasic system using CHCl3 and acetate buffer, and the phosphatidylated product was isolated and characterized as DPP-B1, because we had already done the enzymatic synthesis of DPP-PN and DPP-B2 by PLD in a biphasic system using a mixture of CHCl3 and acetate buffer, as reported previously (10–13). In this reaction, the formation of considerable amounts of DPP-Et having a much higher Rf value than DPPC, DPP-B1, and DPPA-Na on a thin-layer chromatogram developed with CHCl3-MeOH-H2O (3 : 2 : 0.2, v/v/v) was also observed, and confirmed by comparison...
with the authentic DPP-Et on TLC. This showed that PLD catalyzed the transfer reaction of DPP-residue from DPPC to ethanol which was contained as a stabilizer in CHCl₃, and ethanol was a much better acceptor than thiamin. Next, the synthesis of DPP-B₁ via transphatidylation by PLD was done with DPPC and thiamin in a biphasic system using a mixture of ethyl acetate and acetate buffer that we had previously used for the synthesis of four novel phosphatidylated compounds by PLD (14–17). The yield of DPP-B₁ was higher than that obtained in a biphasic system using a mixture of CHCl₃ and acetate buffer. The enzymatic synthesis of other DPP-vitamin derivatives was also performed with PLD in a biphasic system using a mixture of ethyl acetate and acetate buffer. Even when the PLD-catalyzed transphatidylation reaction was done in a biphasic system using a mixture of ethyl acetate and acetate buffer, however, the complete inactivation of PLD by the addition of HCl prior to the extraction of the reaction mixture with a mixture of CHCl₃-MeOH (3 : 1, v/v) was absolutely essential to avoid the formation of DPP-methanol and DPP-ethanol by PLD. In the PLD-catalyzed reaction in a biphasic system using a mixture of organic solvent and the aqueous buffer, transphatidylation and hydrolysis are competitive reactions between the two acceptors, hydroxyl groups of acceptor substrate and water. In the case of small primary alkanols, such as methanol, ethanol, ethalamoline, and serine, the transphatidylation is much faster than the hydrolysis, and the hydrolysis cannot compete with the transphatidylation (1–3). Figure 4 shows that maximum conversion of DPPC to DPP-B₁ by PLD was about 95% after a 6 h-incubation in a biphasic system using a mixture of ethyl acetate and acetate buffer. This value was similar to the conversion reached for phosphatidylethanolamine to phosphatidylglycerol (97%) (26) and for phosphatidylethanol to phosphatidy-L-ascorbic acid (94%) (5). Thiamin was also found to be a very good acceptor for PLD. In the enzymatic synthesis of DPP-TPD, DPP-TPFD, DPP-PaA, and DPP-PaOEt in a semipreparative scale, their isolation, and identification, the excellent reaction conditions for the synthesis of DPP-B₁ as described in Fig. 4 were partially modified under careful consideration of the valuable amounts of TPD, TTFD, and PaOEt kindly supplied by two companies and expensive DPPC. When both the volume of the reaction mixture and the content of PLD were reduced, the incubation time was extended in inverse proportion, while the concentration of the acceptor (vitamin derivative) was 10 times that of the donor (DPPC) in all reaction mixtures. The yields of DPP-TPD, DPP-TPFD, and DPP-PaOEt based on DPPC were 15, 25, and 25%, respectively, but that of DPP-PaA was very low (1.6%). As shown in Fig. 2, residual DPPC in the case of PaA was higher than those in the cases of B₁ and TPD after a 3 h-incubation. The activity of PLD might be inhibited by PaA. Raising the content of PLD may be necessary to elevate the conversion of DPPC to PaA. In order to enhance the yield of DPP-vitamin compounds, the effects of various kinds of organic solvent used in a biphasic system such as diethyl ether, toluene, benzene, and methylene chloride described previously (18), the acceptor (vitamin and its derivative) concentration, and PLD content on transphatidylation reaction will be examined in a future study.

In this paper and previous reports (10–13), we revealed an easy enzymatic synthesis of phospholipid-vitamin derivatives that are lipophilic derivatives of water-soluble vitamins, such as PN, B₂, B₁, PaA, and their derivatives with a primary hydroxyl group. These phospholipid-vitamins with enhanced lipophilicity and enhanced solubility in lipids may be useful for the study of vitamin transport across cellular membranes. A microbial PLD enzyme, the PLD from Streptomyces sp., as described above and reported previously (10–19), has wide acceptor specificity for the transphatidylation reaction. This property may allow the synthesis of structurally more complicated phospholipids of pharmacologically significant compounds with primary hydroxyl groups, aromatic hydroxyl groups, glycosyl groups and so on.

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