Simple transphosphatidylation of phospholipids catalysed by a lipid-coated phospholipase D in organic solvents †

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A lipid-coated phospholipase D (PLD) was prepared by mixing aqueous solutions of PLD and lipids. The lipid-coated PLD showed a high catalytic activity for transphosphatidylation of egg yolk phosphatidylcholine (egg-PC) with alcohols in two-phase benzene–acetate buffer solution. Since both substrates and enzymes are soluble in the organic phase, the reaction proceeded in the benzene phase and the aqueous phase is required to remove the produced choline moiety from the organic phase. When a native PLD was employed instead of the lipid-coated PLD, the reaction was very slow (~1/300 that of the lipid-coated PLD) because the reaction occurs at the interface of the lipophilic substrates and water-soluble enzymes. The transphosphatidylation catalysed by a lipid-coated PLD could be applied in a manner widely independent of the nature of the head groups of the coating lipids and the polarity of the organic solvents, and could be applied also on a large-scale (yield 1–2 g) synthesis to introduce various alcohols, sugars, and nucleic acids at the head groups of phospholipids. We have determined substrate selectivity and Michaelis–Menten kinetics for a lipid-coated PLD and compared the results with those for the native PLD.

Phospholipids are natural biosurfactants that have many applications in the food and pharmaceutical industries.[1,2] Therefore, it is important to develop production methods for phospholipid derivatives in a simple one-step synthesis. Phospholipase D (PLD) is an enzyme that catalyses the hydrolysis of the ester linkage between phosphatidic acid and alcohol moieties of phospholipids. PLD also catalyses the transfer reaction by which the phosphatidic acid moiety is transferred to an acceptor alcohol (transphosphatidylation).[3] Since phospholipid substrates are lipophilic and PLD is soluble only in an aqueous phase, the transphosphatidylation has been carried out in heterogeneous phases such as water–oil emulsions, micelles, and reversed micelles.[4–6] In these heterogeneous systems, reactions occur at the interface of lipophilic substrates and water-soluble PLD; therefore, the enzymic reactivity is lowered, the reaction kinetics are complicated, and hydrolysed by-products (phosphatidic acid) are produced.

We have recently reported a lipid-coated enzyme that is freely soluble in organic solvents and shows high catalytic activity in organic media.[7–11] For example, lipid-coated lipase can catalyse triglyceride syntheses from a 1-monoglyceride and aliphatic acid in homogeneous, dry benzene solution,[8,9] and can act as an enantioselective catalyst for the esterification of racemic alcohols with aliphatic acids in homogeneous dry 'isooctane' (2,2,4-trimethylpentane).[7,10] The catalytic activity of the lipid-coated lipase was 2–100 times higher than those of other systems such as a native lipase in water/oil (w/o) emulsion,[12–14] direct dispersion of powdered lipase,[15–20] and poly(ethylene glycol)-grafted lipase in organic solvents.[21–23] The lipid-coated lipase can prepare esters directly from acids and alcohols; on the other hand, other lipase systems in organic solvents can catalyse only the simple transesterification between activated esters and alcohols.

In this paper, we prepare a lipid-coated phospholipase D (PLD) from *Streptomyces* sp. and apply it as the transphosphatidylation catalyst of egg yolk phosphatidylcholine with alcohols to produce various phospholipid derivatives in organic media. Since both the lipid-coated PLD and phospholipid substrates are soluble only in organic solvents, the reaction is expected to proceed smoothly in the homogeneous organic phase and to avoid the production of the hydrolysed by-product. This system is also suitable for studying enzyme kinetics in homogeneous solution. A schematic illustration of the reaction system is shown in Fig. 1.

Fig. 1 A schematic illustration of a lipid-coated PLD and transphosphatidylation of egg-PC with alcohol in benzene solution in the presence of aqueous buffer

Results and discussion

Effect of coating lipids and origin of enzyme

Lipid-coated PLDs were prepared by mixing an aqueous solution of phospholipase D (from *Streptomyces* sp.) with an acetone solution of dialkyl amphiphiles, and were obtained as precipitates. The obtained lipid-coated PLD was soluble only in most organic solvents. The results are summarized in Table 1.

† Enzyme-lipid complex, part 8. For part 7, see Y. Okahata, F. Fujimoto and K. Ijiro, *J. Org. Chem.*, 1995, in the press.

Table 1 Effects of head-groups of coating lipid on the preparation of a lipid-coated PLD and its transphosphatidylation activity

Lipids	Preparation		
	Yield[a] (mg)	Protein content[b] (wt%)	Enzymic activity[d] [μmol dm^{-3} s^{-1} (mg of protein)$^{-1}$]
$(Me[CH_2]_{11}O_2C)_2$–SO_3^- Na^+ **1**	25.4	7.4 (8.0)[c]	15
$(Me[CH_2]_{11}O)_2P(O)O^-$ Na^+ **2**	20.0	7.5 (7.0)[c]	24
$(Me[CH_2]_{17})_2N^+(Me)CH_2CH_2SO_3^-$ **3**	14.1	8.0 (9.0)[c]	16
$(Me[CH_2]_{11}O_2C)_2$–NHCO–CH(OH)CH(OH)CH(OH)CH(OH)–CH_2OH **4**	3.6	3.9 (2.6)[c]	7.7
$(Me[CH_2]_{11}O_2C)_2$–NH_3^+ Cl^- **5**	7.5	1.8 (1.5)[c]	2.2

[a] Aqueous solutions of PLD (25 mg) and lipids (25 mg) were mixed and precipitates were lyophilized. [b] Obtained from UV absorption of aromatic amino acid residues in the protein. [c] Obtained from C/N ratio of elemental analyses. [d] Initial rates of transphosphatidylation of egg PC (1 mmol dm^{-3}) with butan-1-ol (430 mmol dm^{-3}) in benzene (4 cm^3) at 40 °C catalysed by the lipid-coated PLD (0.05 mg of protein) in the presence of acetate buffer (4 cm^3; 10 mmol dm^{-3}; pH 5.5).

Yields of the lipid-coated PLD as a purified powder were obtained by weighing. The content of protein in the complex was estimated from the C/N ratio of elemental analyses and the UV absorption of aromatic amino acid residues in PLD. The protein contents obtained from both methods were consistent within experimental errors. These values were also consistent with the ratios of lipids and enzymes obtained from gel-permeation chromatography (GPC) measurements. The enzyme activity is shown as an initial rate of transphosphatidylation in the two-phase benzene–acetate buffer solution at 40 °C.

The lipid-coated enzyme was obtained in fair yield independent of the nature of head group charges of the lipid. When anionic (**1**, **2**) and zwitterionic (**3**) lipids were employed, a lipid-coated PLD containing 7–8 wt% of protein was obtained and showed relatively high activity in the transphosphatidylation. In the case of nonionic (**4**) and cationic (**5**) lipids, the protein content and the activity were relatively low. The anionic amphiphile **1** was chosen as the standard coating lipid in the following experiments. The suitable-coating amphiphiles are different for each enzyme: in the case of lipase from *Pseudomonas*, nonionic lipids showed high enzyme activity as a coating lipid in 'isooctane' solution, but this was not the case in coatings with cationic or zwitterionic lipids. Suitable interactions (such as hydrogen bonds and electrostatic interactions) between the enzyme surface and hydrophilic head groups of lipids may be different for each enzyme.

When PLD from cabbage leaves[24] was employed instead of PLD from *Streptomyces* sp., the lipid-coated PLD could be obtained in fair yield and contained ~8% of protein in the complex. However, the lipid **1**-coated PLD from cabbage showed hardly any activity for transphosphatidylation, but catalysed slowly the hydrolysis to phosphatidic acid in the two-phase benzene–buffer solution. The PLD from microorganisms such as *Streptomyces* sp. has been reported to be suitable for transphosphatidylation and physically stable compared with the PLD from plant tissues such as cabbage which shows relatively high hydrolysis activities.[5,25,26] The PLD from *Streptomyces* sp. was chosen as the standard enzyme in the following experiments.

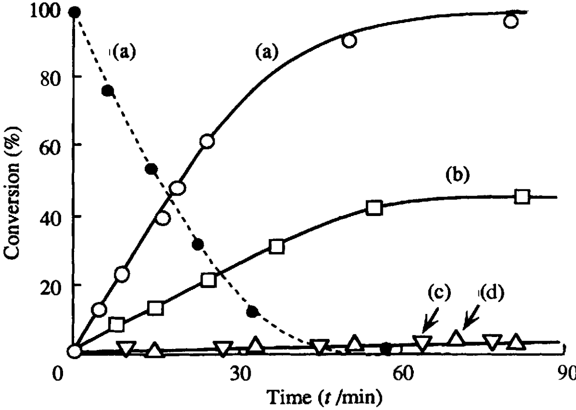


Fig. 2 Typical time courses of transphosphatidylation of egg-PC (1 mmol dm^{-3}) with butan-1-ol (430 mmol dm^{-3}) catalysed by PLD (0.05 mg of protein) at 40 °C. The initial rate of transphosphatidylation obtained from the curve is shown in v_0/μmol dm^{-3} s^{-1} (mg of protein)$^{-1}$. (a) Production of phosphatidylbutanol (PB, ○) and reduction of egg-PC (PC, ●) catalysed by a lipid-coated PLD in a two-phase mixture of benzene (4 cm^3) and acetate buffer (4 cm^3; pH 5.5). (b) Production of phosphatidylbutanol catalysed by a lipid-coated PLD in homogeneous benzene solution (4 cm^3). (c) Production of phosphatidylbutanol catalysed by a native PLD in a two-phase mixture of benzene (4 cm^3) and acetate buffer (4 cm^3; pH 5.5). (d) Production of phosphatidylbutanol catalysed by a native PLD in the dispersed benzene solution (4 cm^3)

Comparison of reaction systems

Typical time courses of transphosphatidylation of egg-PC with butan-1-ol catalysed by PLD are shown in Fig. 2. When the reaction was carried out by using a lipid **1**-coated PLD in two-phase benzene–acetate buffer, the phosphatidylcholine (PC) substrate was converted completely into phosphatidylbutanol

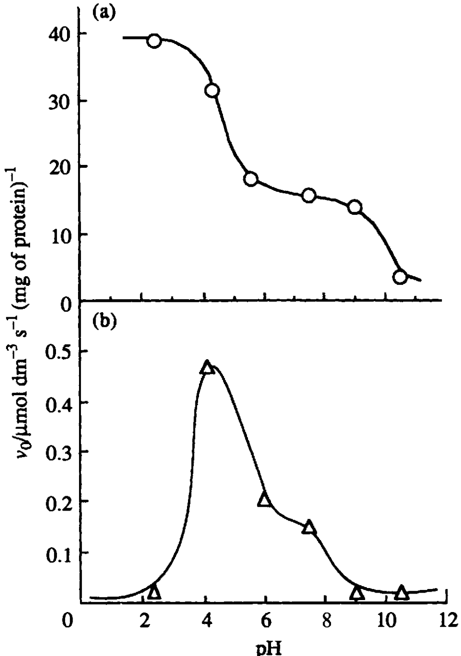


Fig. 3 Effect of pH in the aqueous phase on the transphosphatidylation rates in the two-phase system at 40 °C. (a) The homogeneous reaction catalysed by a lipid-coated PLD and (b) the interfacial reaction catalysed by a native PLD

(PB) within 1 h [curve (a), v_0 = 15 µmol dm^{-3} s^{-1} (mg of protein)$^{-1}$]. The hydrolysed product (phosphatidic acid) was not detected within experimental error, either in the organic or in the aqueous phase. Since both the egg-PC substrate and the lipid-coated PLD are not detected in the aqueous phase during the experiment, the reaction proceeds mainly in the organic phase.

When the reaction was carried out by using a lipid-coated PLD in homogeneous, water-saturated (0.15 wt%) benzene solution without any aqueous buffer phase, the reaction rate was decreased [v_0 = 6.0 µmol dm^{-3} s^{-1} (mg of protein)$^{-1}$] and the transphosphatidylation reaction stopped near 50% conversion [curve (b)]. When the buffer solution (pH 5.5; 10 mmol dm^{-3} acetate) was added after the reaction had reached equilibrium, the reaction again proceeded completely within 1 h.

When a native PLD was employed in the same two-phase reaction system, the transphosphatidylation was extremely slow and most of the unchanged phosphatidylcholine was recovered [curve (c)]. The reaction rate catalysed by a native PLD [curve (a), v_0 = 0.045 µmol dm^{-3} s^{-1} (mg of protein)$^{-1}$] was ~ 1/300 times smaller than that by a lipid-coated PLD [v_0 = 15 µmol dm^{-3} s^{-1} (mg of protein)$^{-1}$]. In the case of a native PLD, the reaction occurs at the interface between lipophilic substrates and the water-soluble enzyme. On the other hand, when the lipid-coated PLD was employed, both phosphatidylcholine substrates and enzymes are solubilized in the same organic phase and the reaction occurs smoothly.

When the reaction was carried out by using a water-soluble, native PLD in benzene without the aqueous buffer phase, the conversion was almost zero [curve (d), v_0 = 0.0038 µmol dm^{-3} s^{-1} (mg of protein)$^{-1}$] because the native PLD is not solubilized and exists as aggregated powder in the organic solution.

Thus, the transphosphatidylation occurs smoothly by using a lipophilic lipid-coated PLD compared with a water-soluble native PLD. Although the reaction proceeds in the organic phase, the aqueous buffer phase is required in order to achieve simple and complete transphosphatidylation. In transphosphatidylations catalysed by a lipid-coated PLD in the two-phase system, the reaction rate increased with increasing stirring speed. The reaction rate decreased with increasing concentration of choline added in the aqueous buffer phase. The conversion was also increased with increasing stirring speed, and decreased with increasing added choline concentration. These results indicate that choline exists as a counter-cation of the phosphatidylbutanol anion in the absence of the water phase and inhibits the following catalytic reaction. Therefore, the aqueous phase is required to remove the choline product from the organic phase to avoid product inhibition.

Table 2 Effect of organic solvents on the transphosphatidylation catalysed by a lipid-coated PLD in a two-phase system at 40 °C[a]

Solvent	Initial rates [v_0/µmol dm^{-3} s^{-1} (mg of protein)$^{-1}$]	Conversion at 2 h (%)
Hexane	16	95
Benzene	15 (21)	95
Chloroform	15 (16)	95
Acetone	15	55

[a] [Egg-PC] = 1 mmol dm^{-3}, [butan-1-ol] = 430 mmol dm^{-3}, [lipid-coated PLD] = 0.1 mg, 0.05 mg of protein, two-phase organic solvent (4 cm^3) and acetate buffer (4 cm^3; 10 mmol dm^{-3}; pH 5.5).

Fig. 3 shows pH-rate profiles in the two-phase reaction catalysed by a lipid-coated PLD solubilized in the organic phase and by a native PLD solubilized in the aqueous phase. The usual bell-shaped pH–rate profile was obtained whose maximum rate appeared at pH 4 in the case of a native PLD,[27] although the reaction rate was very slow due to the interfacial reaction. In the case of the lipid-coated PLD, the reaction rate increased dramatically at low pH. In the neutral pH region, the produced choline exists as a counter-cation of phosphatidylbutanol anion and the release of choline to the aqueous phase may be the rate-determining step. At a pH below 4, choline is easily transferred to the aqueous phase because of the protonation of phosphate anions of the produced phosphatidylbutanol. From these results, it is concluded that the aqueous phase is required to remove the produced choline from the organic phase in the transphosphatidylation catalysed by a lipid-coated PLD in the two-phase system.

Effect of organic solvents in two-phase systems

Table 2 shows the effect of solvent on the transphosphatidylation of egg-PC and butan-1-ol catalysed by a lipid-coated PLD in two-phase systems of the respective organic solvent and acetate buffer. The fair reaction rate and good yield of phosphatidylbutanol after 2 h were obtained independent of the type of solvent. Similarly good results could be obtained in two-phase reactions by using other solvents such as 'isooctane', cyclohexane, toluene, diisopropyl ether, dipropyl ether, and ethyl acetate. In the case of acetone, the reaction system becomes homogeneous and both the reaction rate and yield were slightly decreased compared with other two-phase systems. The initial rates given in parentheses show the enzyme activity after the lipid-coated PLD had been kept in the corresponding solution for 24 h. The enzyme activity was not decreased after incubation in organic solvents for several days.

Substrate selectivity

The lipid-coated PLD system is suitable for studying substrate selectivity of the transphosphatidylation, because the reaction is carried out in the homogeneous phase. Table 3 shows effect of phospholipid head groups on the initial rates of the transphosphatidylation with butan-1-ol in the two-phase system. It clearly shows that PLD can recognize only naturally occurring phosphatidylcholine head groups, and the transphosphatidylation was largely decreased when phospholipids

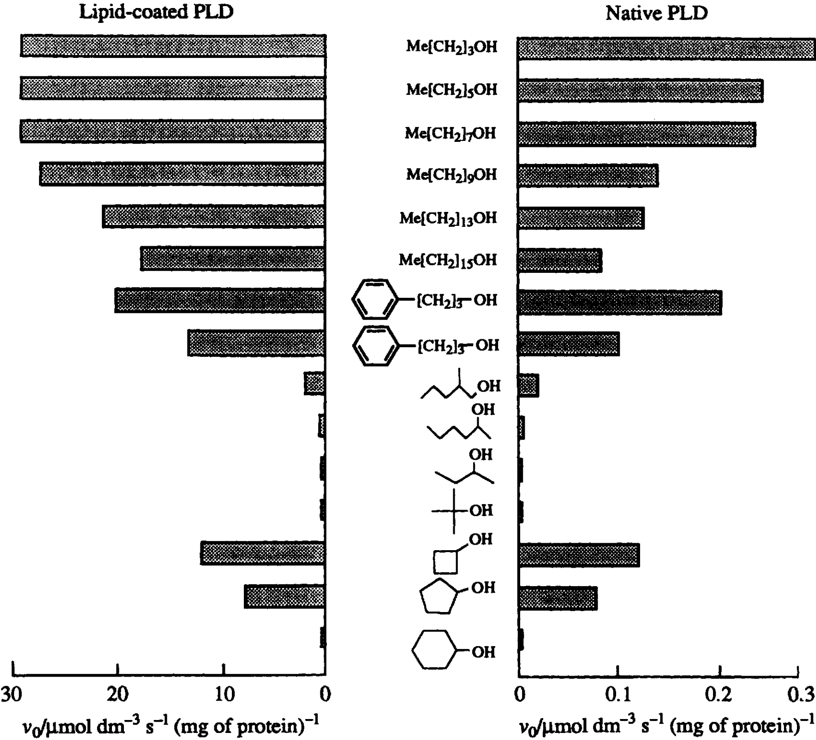


Fig. 4 Substrate selectivity for alcohol moieties of a lipid-coated PLD and a native PLD in the transphosphatidylation of egg-PC in a two-phase mixture of benzene (4 cm^3) and acetate buffer (4 cm^3; pH 5.5)

Table 3 Selectivity for phospholipid substrates on the transphosphatidylation with butan-1-ol catalysed by a lipid-coated PLD in a two-phase system at 40 °C [a]

Phospholipid [b]	Initial rates [µmol dm^{-3} s^{-1} (mg of protein)$^{-1}$]	Conversion at 2 h (%)
Egg-PC	16	95
Porcine brain-PS	0.2	< 5
Egg-PE	0.2	< 5
1,3-Diether-PC [c]	0.2	< 5

[a] [Phospholipid] = 1 mmol dm^{-3}, [butan-1-ol] = 430 mmol dm^{-3}, [lipid-coated PLD] = 0.5 mg, 0.05 mg of protein, two-phase mixture of benzene (4 cm^3) and acetate buffer (4 cm^3; 10 mmol dm^{-3}; pH 5.5). [b] PC: phosphatidylcholine, PE: phosphatidylethanolamine, PS: phosphatidylserine. [c]

$Me[CH_2]_{13}O$ / $Me[CH_2]_{13}O$ $-O-P(=O)(O^-)-O-CH_2CH_2-N^+Me_3$

having phosphatidylserine, phosphatidylethanolamine, and 1,3-diether-type phosphocholine head groups were employed.

Fig. 4 shows effects of chemical structures of acceptor alcohols on the transphosphatidylation of egg-PC in the two-phase system. The lipid-coated PLD showed relatively high transphosphatidylation activity with primary aliphatic alcohols having short linear alkyl chains compared with those having long alkyl chains or a phenyl group. For secondary alcohols, the lipid-coated PLD showed strong substrate selectivity depending on chemical structure: cyclobutanol and cyclopentanol showed high transphosphatidylation compared with other secondary alcohols. When the native PLD was employed under the same reaction conditions, similar substrate selectivity was obtained although the reaction rate was very low due to the interfacial reaction in the two-phase system. This indicates that the coating lipid does not affect the enzyme selectivity but does increase enzyme solubility in the lipophilic organic phase.

Large-scale reactions

We have applied this sytem to produce some practically useful phospholipid derivatives having amino acids, carbohydrates and deoxyribonucleotides, as shown in structures **6–11** with each product yield. These compounds could be obtained and isolated on a large scale (~ 1–2 g) in fair yield (65–85%) in the catalysis of a lipid-coated PLD in the two-phase benzene–acetate buffer (20 + 10 cm^3) system under the following conditions: egg-PC = 2.0 g, alcohol = 7–8 g, lipid-coated PLD = 10 mg. The reaction products were purified by HPLC from the organic layer.

Michaelis–Menten kinetics

The lipid-coated enzyme system is suitable for kinetic measurements because the reaction is carried out in the homogeneous organic phase. Fig. 5 shows typical saturation behaviour of the initial rates when the concentration of hexan-1-ol was changed over the range 10–600 mmol dm^{-3} at a constant concentration of egg-PC (3 mmol dm^{-3}). A similar saturation curve was observed when the egg-PC concentration was changed over the range 0.4–3 mmol dm^{-3} at a constant concentration of hexan-1-ol (200 mmol dm^{-3}).

In the case of two-substrate reactions (S_A and S_B), Michaelis–Menten kinetics can be expressed as eqns. (1)–(3).

$$E + S_A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\,S_A \quad (1)$$

$$E\,S_A + S_B \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\,S_AS_B \quad (2)$$

$$E\,S_AS_B \xrightarrow{k_3} E + P \quad (3)$$

egg-PC

6 Glucose (68%)

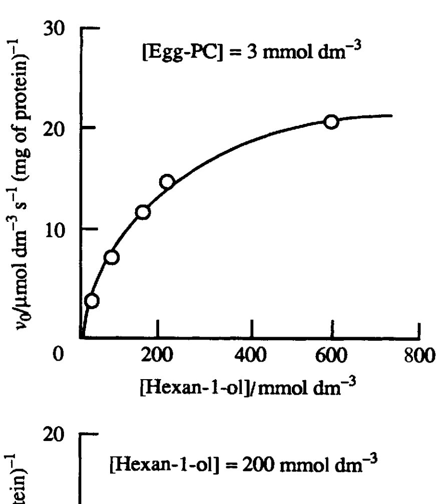
7 Galactose (75%)

8 Serine (75%)

9 Glycerol (85%)

10 Deoxyadenosine (75%)

11 Thymine (70%)



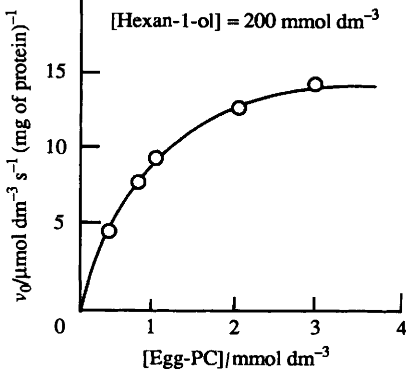


Fig. 5 Michaelis–Menten kinetics of the transphosphatidylation of egg-PC with hexan-1-ol catalysed by a lipid-coated PLD (0.05 mg of protein) in a two-phase system

Table 4 Michaelis–Menten kinetic parameters of the transphosphatidylation of egg-PC with hexan-1-ol catalysed by a lipid-coated PLD

Substrates	K_{mA} (mmol dm^{-3})	K_{mB} (mmol dm^{-3})	k_3 (s^{-1})
S_A = hexanol, S_B = egg-PC	−1.9	−1700	
S_A = egg-PC, S_B = hexanol	2.7	350	4.7

If $[S_A]$ and $[S_B] \gg [E]$, and k_3 is the rate-determining step ($k_3 \ll k_1, k_{-1}, k_2$, and k_{-2}), then eqns. (4)–(6) apply,

$$v = \frac{v_{max}[S_A][S_B]}{K_{mA}K_{mB} + K_{mB}[S_A] + [S_A][S_B]} \quad (4)$$

$$\text{where } v_{max} = k_3[E]_0,\ K_{mA} = \frac{k_1}{k_{-1}} \text{ and } K_{mB} = \frac{k_2}{k_{-2}};$$

when $[S_A]$ is constant and $[S_A] \gg K_{mA}$,

$$\frac{1}{v} = \frac{K_{mA}K_{mB} + K_{mB}[S_A]}{v_{max}[S_A]}\frac{1}{[S_B]} + \frac{1}{v_{max}} \quad (5)$$

when $[S_B]$ is constant and $[S_B] \gg K_{mB}$,

$$\frac{1}{v} = \frac{K_{mA}K_{mB}}{v_{max}[S_B]}\frac{1}{[S_A]} + \frac{K_{mB} + [S_B]}{v_{max}[S_B]} \quad (6)$$

Intercepts and slopes of two Lineweaver–Burk plots of eqns. (5) and (6) obtained from Fig. 5 gave us values for K_{mA}, K_{mB}, and k_3 and the obtained results are summarized in Table 4. When the first binding substrate (S_A) was assumed to be alcohol and the second substrate (S_B) was phospholipid, the obtained values for K_{mA} and K_{mB} were calculated to be negative (−1.9 mmol dm^{-3} and −1710 mmol dm^{-3}, respectively). However, when the S_A was assumed to be egg-PC and the S_B was hexan-1-ol, then positive and reasonable values were obtained (K_{mA} = 2.7 mmol dm^{-3}, K_{mB} = 350 mmol dm^{-3}, and k_3 = 4.7 s^{-1}). Thus, in this two-substrate reaction, the phospholipid is likely to be bound first and strongly to form an acyl–enzyme intermediate and then the alcohol moiety is bound weakly as a second substrate (nucleophile).

Conclusions

The lipid-coated PLD is easily prepared and soluble in most organic media without denaturation for several days. The lipid-coated PLD showed very high transphosphatidylation activity of egg-PC in two-phase benzene–aqueous buffer solution, compared with that catalysed by the native PLD. The reaction proceeded in the organic phase, and the aqueous phase was required to remove the produced choline moiety from the organic phase. The hydrophilic head groups of the coating lipids and the polarity of the reaction media hardly affected transphosphatidylation activity. The substrate selectivity of PLD was not affected by the coating lipids, whose two long alkyl chains act as lipophilic tails to be solubilized in organic media. The lipid-coated PLD will become a useful technique for the efficient introduction of various alcohol moieties to phospholipid head groups. This lipid-coating technique is expected to be widely applicable for other enzymes whose substrates are lipophilic.

Experimental

Lipid molecules

Preparations of dialkyl amphiphiles were reported elsewhere.[28–30] They were confirmed as a single peak by TLC with

a flame-ionization detector (Iatron lab., model TF-10, Tokyo). IR and ^1H NMR spectra of these lipids were consistent with the expected structures. Mps were measured with a polarizing microscope, and a liquid crystalline region is denoted by an arrow.

Sodium 1,2-bis(dodecyloxycarbonyl)ethanesulfonate **1**[28] was prepared from didodecyl maleate and $NaHSO_3$, as granules (70%); ν_{max}(KBr)/cm^{-1} 1725 (C=O) and 1250 (SO_3) (Found: C, 54.8; H, 6. Calc. for $C_{28}H_{53}NaO_7S{\cdot}4H_2O$: C, 53.5; H, 9.80%).

Sodium didodecyl phosphate **2**[28] was prepared from dodecanol and $POCl_3$ in benzene, as granules (78%), mp 33–34 °C; ν_{max}(KBr)/cm^{-1} 1230 (P=O) and 1060 (P–O–C) (Found: C, 62.2; H, 11.5; P. 8.7. Calc. for $C_{24}H_{50}NaO_4P$: C, 61.9; H, 10.7; P, 8.60%).

N-Methyl-*N*,*N*-dioctadecylammonioethanesulfonate **3**[29] was prepared by the stepwise alkylation of 2-(methylamino)-ethanesulfonate by dodecyl bromide, as granules (45%) (Found: C, 68.1; H, 11.5; P, 2.9. Calc. for $C_{27}H_{57}NSO_3$: C, 68.5; H, 12.1; N, 2.74%).

Didodecyl glutamate **5**[30] was prepared from L-glutamic acid and dodecanol in the presence of toluene-*p*-sulfonic acid in 85% yield (Found: C, 72.5; H, 11.5; N, 2.85. Calc. for $C_{29}H_{57}NO_4$: C, 72.0; H, 11.8; N, 2.90%).

Didodecyl *N*-D-glucono-L-glutamate **4**[30] was prepared from didodecyl L-glutamate and D-glucono-1,5-lactone in methanol, as granules (43%), mp 95 → 120 °C (Found: C, 62.6; H, 11.5; N, 2.1. Calc. for $C_{35}H_{67}NO_{10}$: C, 63.5; H, 10.1; N, 2.10%).

Chemical structures of dialkyl amphiphiles **1–5** are shown in Table 1. Egg yolk phosphatidylcholine (egg-PC), alcohol derivatives, and other chemicals were purchased as the finest grade from Tokyo Kasei Co., Tokyo, and Sogo Pharmaceutical Co., Tokyo.

Enzymes
PLD from *Streptomyces* sp. (E.C. 3.1.4.4) as a fine grade, was a gift from Dr. S. Imamura of Asahi Chemicals, Shizuoka, Japan.[5]

Preparation of a lipid-coated PLD
A lipid-coated PLD was prepared in a similar manner to that in previous papers for lipase.[7–10] A hot acetone solution (0.5 cm^3) of the dialkyl amphiphile **1–5** as a typical example was added dropwise to aq. (25 cm^3; 0.01 mol dm^{-3} acetate buffer; pH 5.5) PLD (25 mg) at room temperature, and then the mixture was stirred for 20 h at 4 °C. Precipitates were gathered by centrifugation at 4 °C (5000 rpm; 5 min) and lyophilized. The obtained powder was insoluble in water or any aqueous buffer solution but was freely soluble in most organic solvents.

The protein content in the complex was estimated from both elemental analysis (C, H and N) and the UV absorption of aromatic amino acid residues in the proteins at 280 nm in chloroform solution, in a similar manner to that with an aqeuous solution. Results are summarized in Table 1.

Characterization of a lipid-coated PLD
GPC of a lipid (**1**)-coated PLD showed one peak at the estimated molecular mass of (130 ± 20) × 10^3 (eluent: dichloromethane; detector, UV at 240 nm; molecular mass was calibrated using a standard polystyrene). Since the molecular masses of a native PLD and the lipid **1** were ~ 50 000 and 524, respectively, the lipid-coated PLD was calculated to contain 150 ± 50 lipid molecules per molecule of PLD. It can be roughly estimated from the molecular areas of the lipid **1** (0.45 nm^2) and PLD (diameter, ~ 4 nm) that ~ 150 ± 50 lipid molecules are required to cover the surface of a PLD as a monolayer. These values are consistent with each other and the protein content (7.7 ± 0.3 wt%) in the complex obtained from elemental analyses and UV measurements. Similar results were obtained for the lipid-coated PLD prepared from other amphiphiles.

It should be noted that the coating lipids are stable in that they are not removed from the enzyme surface during GPC in organic solvents. The lipid-coated PLD solubilized in benzene was not extracted to the aqueous phase after washing with the buffer solution (10 mmol dm^{-3} acetate; pH 5.5) several times.

Catalytic activity of a lipid-coated PLD in organic media
A typical procedure is as follows. A benzene solution (4 cm^3) of a lipid (**1**)-coated PLD (0.1 mg, 0.05 mg of protein), egg-PC (1 mmol dm^{-3}) and an excess of butan-1-ol (830 mmol dm^{-3}), and buffer solution (4 cm^3; pH 5.5; 10 mmol dm^{-3} acetate) were gently stirred without disturbance of the interface at 40 °C. Added butan-1-ol (830 mmol dm^{-3}) distributed both in the organic phase and in the aqueous buffer solution, and the concentration of butan-1-ol in the organic phase was determined to be 430 mmol dm^{-3}. With the prescribed time interval, the production of phosphatidylbutanol and the disappearance of phosphatidylcholine in the organic phase were followed by TLC with flame-ionization detection (Iatroscan MK-5, Iatron Laboratories, Inc., Tokyo; TC-11 recorder with integrator; TLC rod, Chromarod-S3). The spot was expanded with two steps using two different solvent systems: $CHCl_3$–MeOH–NH_3 (6:4:0.1) and then hexane–diethyl ether (6:4). Identification and quantification of the substrates and the products were performed by comparing the R_f-value and the peak area with those of authentic samples.

The egg-PC substrates and the phosphatidylbutanol products were detected only in the organic phase, together with the lipid-coated PLD, and the choline products were detected only in the aqueous phase (NMR measurements).

Large-scale catalytic reactions
A benzene solution (20 cm^3) of a lipid-coated PLD (10 mg, 0.5 mg of protein), egg-PC (2.0 g; 15 mmol dm^{-3}), and an excess (6–8 g; 1 mol dm^{-3}) of an alcohol (glucose, galactose, serine, glycerol, deoxyadenosine, or thymine) and a buffer solution (10 cm^3; pH 5.5; 10 mmol dm^{-3} acetate) were gently stirred at 40 °C. After one day, the organic phase was evaporated and the residue was purified by HPLC (Instrument: TOSO, Co., model CCDP; column: TSK-OTS, 20 × 300 mm; eluent: MeCN–water, 1 : 1; detector: UV at 240 nm). The transphosphatidylation products were isolated in 65–85% yield (1.5–1.8 g) as shown in structures **6–11**. NMR and IR spectra of these products were consistent with the expected structures, and their analyses are summarized below.

1,2-Diacyl-3-glycerophospho-6-glucose **6** (1.51 g, 68%), ν_{max}(neat)/cm^{-1} 3450 (OH) and 1720 (C=O) (Found: C, 63.5; H, 10.2; P, 3.4. Calc. for $C_{45}H_{81.5}O_{13}P$: C, 62.8; H, 9.50; P, 3.60%).

1,2-Diacyl-3-glycerophospho-6-galactose **7** (1.68 g, 75%), ν_{max}(neat)/cm^{-1} 3450 (OH) and 1720 (C=O) (Found: C. 63.2; H, 9.8; P, 3.7. Calc. for $C_{45}H_{81.5}O_{13}P$: C, 62.8; H, 9.50; P, 3.60%).

1,2-Diacyl-3-glycerophosphoserine **8** (1.53 g, 75%), ν_{max}(neat)/cm^{-1} 1750 (C=O) and 1720 (C=O) (Found: C, 64.0; H, 10.0; N, 1.9; P, 4.0. Calc. for $C_{42}H_{76.5}NO_{10}P$: C, 64.2; H, 9.74; N, 1.80; P, 3.90%).

1,2-Diacyl-3-glycerophosphoglycerol **9** (1.70 g, 85%), ν_{max}-(neat)/cm^{-1} 3460 (OH) (Found: C, 65.8; H, 10.2; P, 4.2. Calc. for $C_{42}H_{76.5}O_{10}P$: C, 65.3; H, 9.90; P, 4.00%).

1,2-Diacyl-3-glycerophospho-5′-deoxyadenosine **10** (1.78 g, 75%); δ_H($CDCl_3$) 2.5 (2 H, 2-H_2 of deoxyribose) and 8.3 (1 H, 8-H of adenosine) (Found: C, 63.8; H, 10.0; N, 7.8; P. 3.3. Calc. for $C_{49}H_{83.5}N_5O_9P$: C, 64.2; H, 9.10; N, 7.60; P, 3.40%).

1,2-Diacyl-3-glycerophospho-5′-thymine **11** (1.68 g, 70%), δ_H($CDCl_3$) 1.8 (3 H, Me of thymidine) and 2.5 (2 H, 2′-H_2 of deoxyribose) (Found: C, 64.5; H, 10.2; N, 2.8; P, 3.3. Calc. for $C_{49}H_{84.5}N_2O_{12}P$: C, 63.7; H, 9.10; N, 3.00; P, 3.40%).

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