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Production of 1,2-Didocosahexaenoyl Phosphatidylcholine by Bonito Muscle Lysophosphatidylcholine/Transacylase

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1,2-Didocosahexaenoyl phosphatidylcholine (PC), which has highly unsaturated fatty acid at both *sn*-1 and *sn*-2 positions of glycerol, is a characteristic molecular species of bonito muscle. To examine the involvement of a *de novo* route in its synthesis, the molecular species of phosphatidic acid (PA) were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using a 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato dizinc(II) complex, a novel phosphate-capture molecule. However, 1,2-didocosahexaenoyl species could not be detected. Next, 1,2-didocosahexaenoyl PC synthesis by the cytosolic lysophosphatidylcholine (LPC)/transacylase was examined using endogenous LPC from bonito muscle, in which the 2-docosahexaenoyl species is abundant. The LPC/transacylase synthesized 1,2-didocosahexaenoyl PC as the most abundant molecular species. For further characterization, the LPC/transacylase was purified to homogeneity from the 100,000 × *g* supernatant of bonito muscle. The isolated LPC/transacylase is a labile glycoprotein with molecular mass of 52 kDa including a 5-kDa sugar moiety. The LPC/transacylase showed a PC synthesis (transacylase activity) below and above the critical micelle concentration of substrate LPC, and fatty acid release (lysophospholipase activity) was always smaller than the transacylase activity, even with a monomeric substrate. These results suggest that the LPC/transacylase is responsible for the synthesis of 1,2-didocosahexaenoyl PC.

Key words: 1,2-didocosahexaenoyl PC, lysophospholipase-transacylase, molecular species, phosphatidic acid, Phos-tag®.

Abbreviations: LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLA_1, phospholipase A_1; PLA_2, phospholipase A_2; TOF/MS, time-of-flight mass spectrometry; Phos-tag®, 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato dizinc(II) complex, $^{68}Zn_2L^{3+}$.

Lysophosphatidylcholine (LPC) occurs at concentrations as high as 10% in fresh bonito muscle. It occurs in two forms, 1-acyl LPC and 2-acyl LPC, the former produced by the action of phospholipase A_2 (PLA_2) and the latter by phospholipase A_1 (PLA_1). We have reported that the structure of LPC from bonito muscle was composed mainly of 2-acyl LPC, in which highly unsaturated fatty acids, such as eicosapentaenoic acid and docosahexaenoic acid were esterified (*1*). In this connection, we detected PLA_1 activity (*2*), and a calcium-independent PLA_1 with a molecular mass of 71.5 kDa was purified from the 100,000 × *g* supernatant fraction (*3*).

Glycerophospholipids from fish muscle include unusual molecular species that contain highly unsaturated fatty acids at both *sn*-1 and *sn*-2 positions (*4*, *5*), and substantial amounts of 1,2-didocosahexaenoyl PC were detected in bonito muscle (*6*). Three routes for the synthesis of 1,2-didocosahexaenoyl PC can be considered: a de novo route and two remodeling routes using LPC. Accordingly, we examined the possible routes for the synthesis of 1,2-didocosahexaenoyl PC by utilizing 2-acyl LPC from muscle. Incorporation of docosahexaenoic acid into the *sn*-1 position of 2-acyl LPC was first examined using bonito muscle microsomes in the presence of ATP and CoA. However, muscle microsomal acyltransferase carried docosahexaenoic acid exclusively to the *sn*-2 position of 1-acyl LPC, which is present as a minor counterpart of LPC in microsomal preparations. Next, we examined PC formation from 2-acyl LPC in the cytosol fraction, and a novel calcium and CoA-independent transacylase activity was detected (*6*). The enzymatic conversion of two molecules of LPC to PC was first described by Erbland and Marinetti using rat liver preparation free from cell particles (*7*). This conversion was performed by a cytosolic lysophospholipase-transacylase, which catalyzes the transesterification of fatty acid from one LPC to another LPC as well as the degradation of LPC to free fatty acid and glycerophosphocholine. Thereafter, the activity was found in cytosols from rat lung (*8*) and stomach (*9*), and rabbit heart (*10*), and the heart lysophospholipase-transacylase was purified to near

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homogeity (*11*). Recently, rat liver cytosolic lysophospholipase-transacylase was purified and found to exhibit both hydrolytic and transacylase activities, their ratio being 1: 0.3 under the standard assay conditions (*12*). These lysophospholipase-transacylase activities were assayed using 1-palmitoyl LPC as substrate. The results suggested lysophospholipase-transacylase is important, at least in part, in the synthesis of a disaturated molecular species of phospholipid, 1,2-dipalmitoyl PC, or in scavenging the toxic 1-acyl LPC.

In the present paper, we analyzed PA molecular species to examine the possibility of de novo synthesis, then characterized the LPC/transacylase from bonito muscle. A possible mechanism of production of molecular species having highly unsaturated fatty acid at both *sn*-1 and *sn*-2 positions of glycerol, such as 1,2-didocosahexaenoyl PC, is discussed.

MATERIALS AND METHODS

Materials—PLA_1 was prepared from bonito muscle as described (*3*). PLA_2 from *Crotalus adamanteus* venom and phospholipase C from *Bacillus cereus* were the product of Sigma Chemicals (St. Louis, MO, USA). Phospholipase D was prepared from acetone powder of cabbage leaves (*13*). Standard PC and phosphatidylethanolamine (PE) were prepared from egg yolk by successive silicic acid column and thin-layer chromatographies, and phosphatidic acid (PA) was prepared by the hydrolysis of egg yolk PC with phospholipase D. 1,2-Di[1-^{14}C]oleoyl PC (118 mCi/mmol) was purchased from Amersham International (Amersham, UK), and 1,2-dioleoyl PC was from Avanti Polar Lipids (Birmingham, AL, USA). 1-[1-^{14}C]Oleoyl LPC with specific activity of 200 cpm/nmol and 2,000 cpm/nmol was prepared by PLA_2 treatment of the corresponding 1,2-di[1-^{14}C]oleoyl PC diluted with 1,2-dioleoyl PC (*6*). 1-Palmitoyl-2-[1-^{14}C]linoleoyl PC and 1-palmitoyl-2-[1-^{14}C]linoleoyl PE (58 mCi/mmol) were purchased from Du Pont–New England Nuclear (Boston, MA, USA) and Amersham International, and diluted with 1-palmitoyl-2-linoleoyl PC and egg yolk PE to specific activities of 200 cpm/nmol, respectively. They were treated with PLA_1 and the resulting 2-[1-^{14}C]linoleoyl LPC and LPE were used for the transacylation assay (*3*). 2-Docosahexaenoyl LPC was chemically prepared from beef heart choline phosphoglyceride as described previously (*2*). 1-Palmitoyl-2-linoleoyl PC and docosahexaenoic acid were the products of Serdary Research Laboratories (Ontario, Canada). Pre-coated thin-layer plates (Silica gel 60) were the product of Merck (Darmstadt, Germany). DEAE-Cellulofine was obtained from Seikagaku Kogyo (Tokyo). Q-Sepharose, Phenyl Sepharose CL-4B and Sephadex G-150 were from Pharmacia Fine Chemicals (Uppsala, Sweden). Bio-Gel HT Hydroxylapatite and Ether Toyopearl were from Bio-Rad Laboratories (Hercules, CA, USA) and Tosoh (Tokyo). Ultrafree-15 Centrifugal Filter Device was from Millipore (Bedford, MA, USA). Peptide: *N*-glycosidase F was the product of New England Biolabs (Beverly, MA, USA). 3,5-Dinitrobenzoyl chloride was from Dojindo Laboratories (Kumamoto). 3,5-Dimethoxycinnamic acid and 2,4,6-trihydroxy-acetophenone were from Aldrich (Milwaukee, WI, USA). Dinuclear single zinc-isotope (II) complex 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato dizinc (II) complex, $^{68}Zn_2L^{3+}$, Phos-tag®, was obtained from the NARD Institute Ltd. (Hyogo) and MANAC Incorporated group (Hiroshima).

Preparation of the LPC/Transacylase—Bonito *Euthynnus pelamis* (Linnaeus) was obtained from a local market in November 1999 and 2000. Preparation of the cytosolic fraction followed the procedure as described using fresh bonito muscle (*6*). All procedures were carried out at 4°C. The 100,000 × *g* supernatant fraction was applied to a DEAE-Cellulofine column. The LPC/transacylase was eluted just behind the PLA_1 (*6*). The threefold increase in total activity by passing through the DEAE-Cellulofine column is gained by removal of endogenous lipids, such as free fatty acids, which are inhibitory to the LPC/transacylase activity (data not shown). To separate PLA_1 completely, the active fraction of LPC/transacylase was applied to a Q-Sepharose column and eluted with a NaCl gradient. The active fraction of LPC/transacylase was concentrated in a Millipore ultrafiltration cell to approximately 100 ml, then added to an equal volume of a basic buffer, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA and 10% glycerol containing 3.9 M $(NH_4)_2SO_4$. The solution was applied to an Ether-Toyopearl column. The activity was recovered in the through fraction, which was then applied to a Phenyl Sepharose column. The LPC/transacylase activity was eluted with a decreasing gradient of $(NH_4)_2SO_4$ concentration. The active fractions were pooled and concentrated by ultrafiltration. The condensate was applied to a Sephadex G-150 column, previously equilibrated with the buffer containing 0.3 M NaCl. The active fractions were pooled and concentrated by ultrafiltration, and the concentrate was dialyzed overnight against 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 10% glycerol. The dialysate was applied to a Hydroxylapatite column and eluted with a phosphate gradient (10–400 mM).

Preparation of Phosphatidic Acid—Lipid was extracted from bonito muscle by a modification of the Bligh and Dyer method (*14*), in which the upper phase was acidified with 2 N HCl to pH 2. Total lipid containing 1.6 mg of lipid phosphorus was applied to a preparative thin-layer plate and developed with a solvent system of chloroform: acetone:methanol:acetic acid:water (50:20:10:13:5, v/v). The spots corresponding to standard PA, PC and PE were scraped off and extracted by a modification of Bligh and Dyer's procedure in acidic conditions. The extracts of PA and PC fractions were applied separately to thin-layer plates and developed with chloroform:methanol:28% ammonia (60:35:8, v/v). PE was prepared by thin-layer chromatography using a solvent system of chloroform: methanol:water (65:35:6, v/v). The isolated PC and PE, corresponding to 40 μg and 20 μg of lipid phosphorus, respectively, were hydrolyzed with 5.6 mg of protein of phospholipase D in 2 ml of biphasic system of 10 μmol acetate buffer (pH 5.8) and diethylether containing 10 μmol $CaCl_2$. The mixture was incubated at 30°C for 2 h with vigorous shaking. After evaporating diethylether, lipid was extracted by a modification of Bligh and Dyer's procedure. The extract was applied to a thin-layer plate, and the PA formed was isolated by developing with a solvent system of chloroform:methanol:28% ammonia (60: 35:8, v/v). From the starting amounts of PC and PE, more

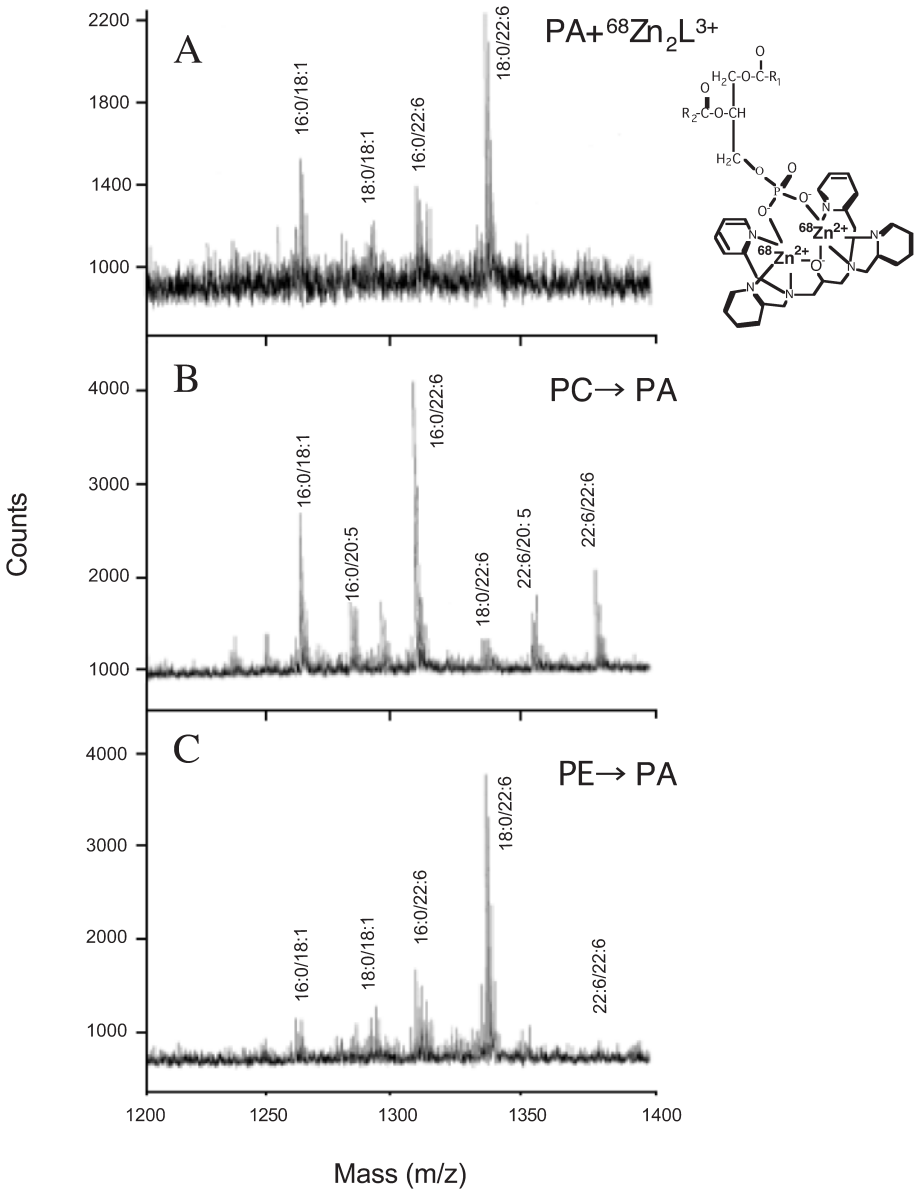

Fig. 1. **MALDI-TOF mass spectra of phospholipids from bonito muscle.** A, PA; B, PC; and C, PE. PC and PE were analyzed as Phos-tag® derivatives after conversion to PA. The formula weight of 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato diz-inc(II) complex, $^{68}Zn_2L^{3+}$ is 589.

than 50% and 20% of lipid phosphorus were recovered as PA, respectively.

LPC/Transacylase Assay—PC formation by the LPC/transacylase proceeded optimally at 20°C and pH 6.5, and the transacylation activity was the same with either 1-oleoyl LPC or 2-oleoyl LPC as substrate (*6*). Previously, the reaction mixture was vigorously shaken with a Taitec TC-8 concentrator at maximum speed, but it was found that the PC formation was twice as abundant when the mixture was not shaken. Accordingly, the standard reaction mixture consisted of 50 nmol of 1-[1-^{14}C]oleoyl LPC (200 cpm/nmol), 250 μmol of Tris-HCl (pH 6.5), and enzyme in a total volume of 1 ml, and it was allowed to stand at 20°C during the reaction. After 30 min, the reaction was stopped by addition of chloroform:methanol (1:1, v/v), and lipids were extracted by the method of Bligh and Dyer (*14*). The extract was applied to a thin-layer plate and developed with the solvent system of chloroform: methanol:water (65:35:6, v/v). The regions corresponding to LPC, PC and free fatty acid were scraped off the plate and radioactivities were counted.

Peptide: N-Glycosidase Treatment—The LPC/transacylase from the Hydroxylapatite column (2.5 μg) was incubated with the peptide: *N*-glycosidase F (500 units) at 37°C overnight (*15*). An aliquot of the mixture was subjected to SDS-PAGE and the remainder to TOF/MS analysis to determine the molecular weight.

Reversed-Phase High-Performance Liquid Chromatography—PC synthesized by the LPC/transacylase was dephosphorylated with phospholipase C, and the resulting diglycerides were converted to dinitrobenzoyl derivatives. They were separated with a ODS 12-T column using a mixture of acetonitrile:2-propanol (90:10, v/v) as an eluting solution (*2*).

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry—The LPC/transacylase from the preparation of Hydroxylapatite column and its degly-

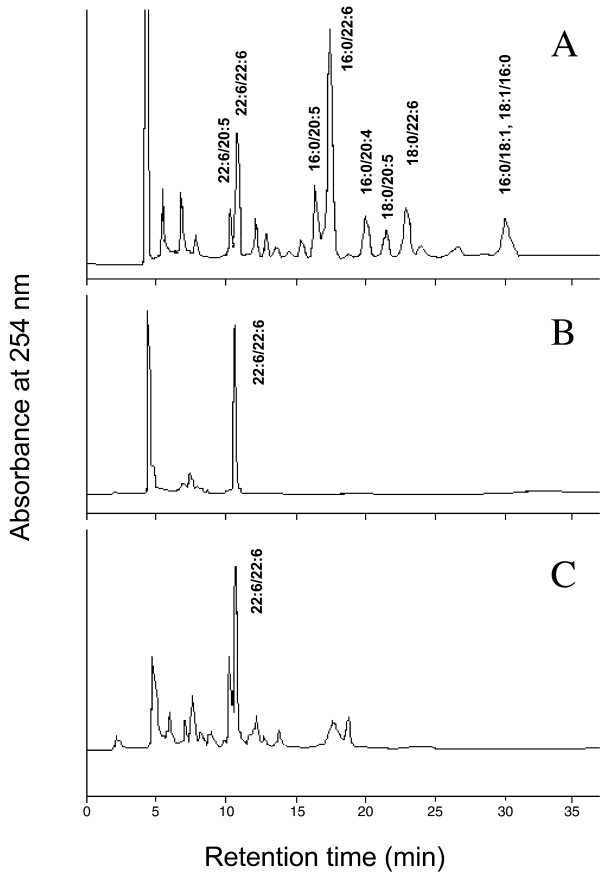

Fig. 2. **High performance liquid chromatograms of the transacylation products by the LPC/transacylase.** The chromatograms are: A, PC from bonito muscle; B, transacylation product using 2-docosahexaenoyl LPC; and C, transacylation product using bonito muscle LPC. The fatty acid composition of LPC was: 14:0, 1.5%; 16:0, 10.5%; 18:0, 3.8%; 18:1(n-9), 6.0%; 18:1(n-7), 1.7%; 20:4 (n-6), 3.9%; 20:5 (n-3), 11.5%; 22:5 (n-3), 3.0%; and 22:6 (n-3), 55.4%. The amount of LPC was 300 nmol, and 200 μg of the preparation from the DEAE-Cellufofine column was used for the synthesis.

cosilated form were analyzed for molecular weight using 3,5-dimethoxycinnamic acid as a matrix (*3*). PA from bonito muscle was analyzed as a Phos-tag® adduct (*16*). PC and PE isolated from bonito muscle were also analyzed after convertsion to PA. The PA preparations analyzed corresponded to 1 μg of lipid phosphorus and were dissolved in 0.1 ml of 10 mM Tris-borate buffer (pH 8.0). Five μl of $^{68}Zn_2L^{3+}$ solution (0.1 mM) was mixed with 5 μl of the PA solution, and 0.5 μl of the mixed solution was spotted on the sample plate. Immediately, 0.5 μl of 2,4,6-trihydroxy-acetophenone solution (10 mg/ml in acetonitrile) was layered on top. The solution of matrix/analyte on the sample plate was allowed to dry for a few minutes. The matrix/analyte crystal formed was analyzed with a PerSeptive Voyager DE-STR TOF mass spectrometer and spectra were obtained in the positive ion mode using a nitrogen laser.

Estimation of Critical Micelle Concentration—The critical micelle concentration of 1-oleoyl LPC was estimated with 2-*p*-toluidinylnaphthalene-8-sulfonate as a fluorescent probe (*17*). Fluorescent spectra were obtained using a JASCO Corporation Spectrophotometer FP-750. The excitation wavelength was 366 nm, and emission was measured at 480 nm.

Other Methods—SDS–polyacrylamide gel electrophoresis was carried out by the method of Laemmli (*18*). The phosphorus concentration was determined according to the procedure of Bartlett (*19*). The protein concentration was tentatively determined by measuring absorbance at 280 nm using an absorption coefficient of 1% LPC/transacylase of $E = 10\ cm^{-1}$.

RESULTS

Analysis of Phosphatidic Acid Molecular Species—Bonito muscle PC is characterized by an abundance of 1,2-didocosahexaenoyl species (*6*). To examine the possibility of de novo synthesis of 1,2-didocosahexaenoyl PC, analysis of PA molecular species is needed. To this end, we developed a mass spectrometric method to analyze PA after converting it to the Phos-tag® derivative. At the same time, PC and PE were converted to PA, and their molecular species were analyzed for direct comparisons with that of PA. As shown in the mass spectrum of Fig. 1A, the most abundant species in PA was 1-stearoyl-2-docosahexaenoyl species followed by 1-palmitoyl-2-oleoyl species, which is thought to be a typical molecular species formed by a de novo pathway. 1-Stearoyl-2-docosahexaenoyl species was the most abundant species in muscle PE (Fig. 1C), and 1-palmitoyl-2-oleoyl species was main species next to 1-palmitoyl-2-docosahexaenoyl species in muscle PC (Fig. 1B). However, molecular species containing highly unsaturated fatty acid at both *sn*-1 and *sn*-2 positions could not be detected in PA (Fig. 1A). Using this analytical technique, we also confirmed our previous findings that 1,2-didocosahexaenoyl species was major constituent in addition to 1-palmitoyl species in PC (Fig. 1B), and that this species was scarcely detected in PE (Fig. 1C).

PA, an intermediate in phospholipid biosynthesis, did not include 1,2-didocosahexaenoyl species, suggesting that de novo synthesis of didocosahexaenoyl PC did not occur. Previously, we found a novel transacylase to form PC from two molecules of LPC in 100,000 × *g* supernatant fraction of bonito muscle. Accordingly, 1,2-didocosahexaenoyl PC synthesis by the LPC/transacylase was examined as an alternative candidate.

Formation of 1,2-Didocosahexaenoyl PC by the LPC/Transacylase—As shown in the HPLC chromatogram of Fig. 2A, the molecular species of bonito muscle PC are characterized by the abundance of 1,2-didocosahexaenoyl PC. When 2-docosahexaenoyl LPC was employed, the LPC/transacylase synthesized 1,2-didocosahexaenoyl PC (Fig. 2B), as was previously confirmed by the mass spectrometric analysis (*6*). Using an endogenous muscle LPC, which consists of more than 50% docosahexaenoic acid, the LPC/transacylase synthesized 1,2-didocosahexaenoyl PC as the most abundant molecular species (Fig. 2C).

Effect of Substrate Head Group on the LPC/Transacylase Activity—To examine the specificity of LPC/transacylase for the polar head group of the substrate, 2-[1-^{14}C]linoleoyl LPC and 2-[1-^{14}C]linoleoyl LPE were employed. The amount of PC formed by the transacylation activity was far higher than the amount of fatty acid released by the lysophospholipase activity for 2-[1-

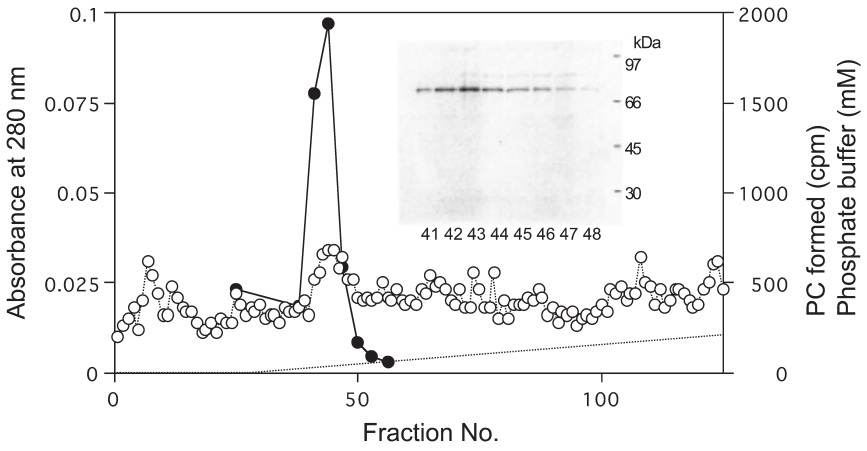

Fig. 3. **Elution profile of the LPC/transacylase on Hydroxylapatite column chromatography.** Pooled active fractions from gel-filtration on a Sephadex G-150 column were concentrated and applied to a Hydroxylapatite column. LPC/transacylase activity (closed circles) and protein (open circles) were monitored at 280 nm. The dotted line is the concentration of phosphate. Inset: fractions 41–48 (5-μl aliquots) were analyzed on a 10% acrylamide/SDS gel visualized with Coomassie Brilliant Blue.

^{14}C]linoleoyl LPC. When the head group of lysophospholipid changed from choline to ethanolamine, the formation of PE from two molecules of 2-[1-^{14}C]linoleoyl LPE dropped steeply (Table 1). This result indicates that the LPC/transacylase discriminates between head groups of lysophospholipid for the transacylation, resulting in a high abundance of 1,2-didocosahexaenoyl species in PC (Fig. 1B) and a low abundance in PE (Fig. 1C).

Purification of the LPC/Transacylase—The LPC/transacylase was isolated after six chromatographic steps, and almost 18,000-fold purification with 15% recovery was achieved. Namely, 620 μg of the LPC/transacylase with a specific activity of 97.2 nmol/mg/min was isolated from 75.1 g of 100,000 × *g* supernatant protein with a specific activity of 5.3 pmol/mg/min (data not shown). SDS/PAGE of the fraction from the Hydroxylapatite column showed that the LPC/transacylase migrated just above the standard molecular marker, bovine serum albumin (Fig. 3, inset) and the TOF/MS analysis demonstrated that of the combined fractions from 41 to 43 gave a single charged molecular ion peak at 52.6 kDa (data not shown). The fact that the molecular masses of the LPC/transacylase determined by SDS-PAGE and by TOF/MS are different suggested that the LPC/transacylase might be a glycoprotein. Accordingly, the isolated LPC/transacylase was treated with peptide: *N*-glycosidase F, which cleaves sugar moieties attached to asparagine residues of protein. As shown in Fig. 4, the LPC/transacylase treated with peptide: *N*-glycosidase F gave a slower migrating band than the intact LPC/transacylase in SDS/PAGE (Fig. 4C), and TOF/MS mass spectra indicated a shift in molecular mass from 52 kDa to 47 kDa. The latter $[M+H]^+$ ion is sharper than the ion from intact LPC/transacylase, indicating that molecular heterogeneity due to glycosilation is reduced (Fig. 4, A and B).

Effect of LPC Concentration on the LPC/Transacylase Activity—The critical micelle concentration of 1-oleoyl LPC was estimated with 2-*p*-toluidinylnaphthalene-8-sulfonate. Enhancement of the fluorescence intensity of 2-*p*-toluidinylnaphthalene-8-sulfonate at 480 nm as a function of the concentration of 1-oleoyl LPC was plotted, and two straight lines could be drawn, indicating the critical micelle concentration was about 5 μM. As shown in Fig. 5, LPC/transacylase activity was also changed around this concentration: it increased gradually up to 5 μM, then increased steeply up to 20 μM. Interestingly, the formation of free fatty acid due to lysophospholipase activity was less than the PC formation by the transacylation even with a monomeric substrate LPC.

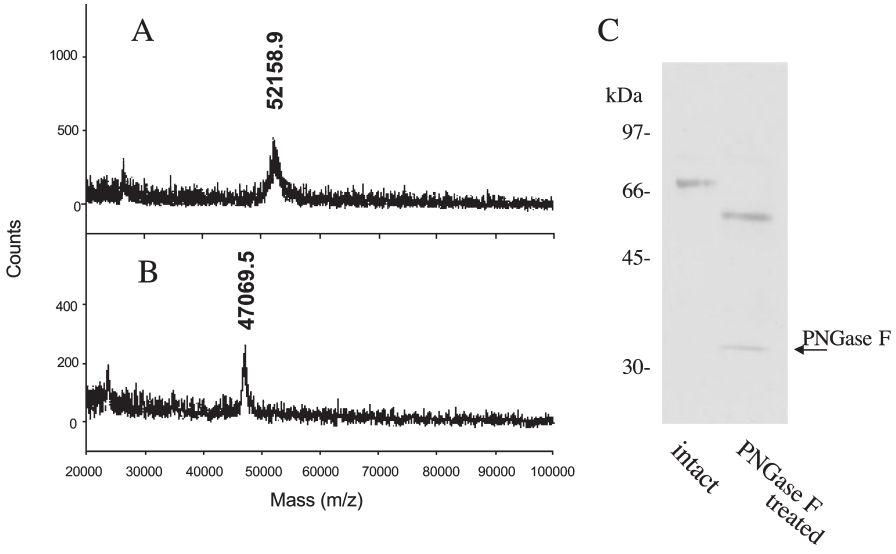

Fig. 4. **Molecular mass shift by a treatment of the LPC/transacylase with peptide: *N*-glycosidase F.** To the purified LPC/transacylase from the Hydroxylapatite column (2.5 μg), peptide: *N*-glycosidase F (500 units) was added, and the mixture was incubated at 37°C overnight. A part of the mixture was subjected to SDS-PAGE (C) and the remainder to TOF/MS analyses for molecular weight determinations (A, B). The ions appearing in the range of 20–30 kDa are double-charged molecular ions of the LPC/transacylase.

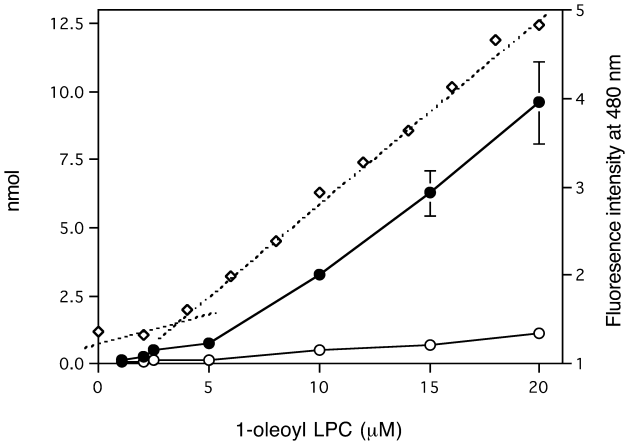

Fig. 5. **LPC/transacylase activity on increasing concentration of substrate.** The assay was perfomed as described in "MATERIALS AND METHODS" using 2,000 cpm/nmol of 1-[1-^{14}C]oleoyl LPC at the concentration of 5 μM, and 200 cpm/nmol of 1-[1-^{14}C]oleoyl LPC at concentrations up to 20 μM. LPC/transacylase activity (closed circles), lysophospholipase activity (open circles) and fluorescence intensity (open squares) were mesured. Because the purified LPC/transacylase was labile, values are mean ± SD of three determinations using 8 μg of the preparation from the Phenyl-Sepharose column.

DISCUSSION

The fatty acid composition of lipids from highly migratory fishes such as bonito and tuna is characterized by high concentrations of docosahexaenoic acid. Because the seasonal variation of docosahexaenoic acid concentration is small, it is speculated that these fishes accumulate docosahexaenoic acid selectively (*20*, *21*). Through analyses of bonito muscle lipids (*1–3*), it was found that phospholipids were 30 times more abundant than triglyceride, and PC accounted for about 60% of phospholipids. The most abundant fatty acid in PC was docosahexaenoic acid, and one third of the docosahexaenoic acid occupied the *sn*-1 position. As far as molecular species are concerned, species having highly unsaturated fatty acids at both the *sn*-1 and *sn*-2 positions of glycerol were detected (*6*). These molecular species were initially found in retina (*22*). Thereafter, 1,2-diarachidonoyl PC was found in rat testis (*23*) and human neutrophils (*24*). This species was found to be continually synthesized de novo, but it does not accumulate to levels above 1% of total PC by the remodeling pathway in mouse macrophage (*25*).

Recently, we found PLA_1 activity in bonito muscle and characterized the strict regiospecificity of multiple enzyme activities at the *sn*-1 position of substrate glycerides (*2*, *3*). Thereafter, we found the LPC/transacylase activity, which forms PC from two molecules of LPC without utilizing ATP (*6*). According to the classification of lysophospholipase-transacylase by Waite (*26*), three types exist: those that are hydrolytic only, those that transacylate in micelles, and those that transacylate with monomers of substrate. A small-form lysophospholipase with molecular mass of 24 kDa from rat liver (*27*) belongs to the first type, and large-form lysophospholipases including one of 50 kDa from rat lung (*8*) and another of 60 kDa from rat liver (*12*) belong to the second type. Of the last type, a lysophospholipase-transacylase with molecular mass of 63 kDa from rabbit myocardium is known (*11*). The lysophospholipase-transacylases are located in cytosolic fraction, and in no case was the activity of transacylase higher than that of lysophospholipase. In contrast, the LPC/transacylase from bonito muscle is a 52-kDa glycoprotein (Fig. 4) and unique in catalyzing transacylation without a significant production of free fatty acid, even with a monomeric substrate of LPC at below the critical micelle concentration (Fig. 5), at which the lysophospholipase activity is predominant (*8*). As far as which fatty acid selectively to transfer is concerned, the LPC/transacylase does not discriminate strictly in terms of the degree of unsaturation or the position of fatty acid esterified in LPC (*6*). However, the form of LPC in muscle was 2-LPC and the fatty acid mainly composed was docosahexaenoic acid, which would be due to the presence of PLA_1 in muscle (*1–3*). Concerning the intracellular distribution of the LPC/transacylase, glycoprotein has scarcely been observed in cytosol, and if present, it is *O*-glycosylated. The cytosol fraction was prepared by the sequential centrifugation, and the 100,000 × *g* supernatant was used as a source of the enzyme. It is impossible to keep bonito at hand. We got bonito from a local fish market as soon as possible after its capture along the Pacific coast of Japan. However, a few days had passed in every case. Therefore, it is possible that a component from an organelle might be included in the 100,000 × *g* fraction.

Table 1. **Effect of substrate head group on the LPC/transacylase activity.**

	2-[1-^{14}C]linoleoyl LPC (nmol)	2-[1-^{14}C]linoleoyl LPE (nmol)
PC or PE formed	5.27 ± 0.30	0.33 ± 0.14
Fatty acid released	0.80 ± 0.05	0.36 ± 0.05

Fifty nanomoles of 2-[1-^{14}C]linoleoyl LPC and 2-[1-^{14}C]linoleoyl LPE were employed as substrates. The enzyme preparation from the Ether-Toyopearl column was used, and the amount was 180 μg. Values are mean ± SD of three determinations.

PA is an intermediate of phospholipid biosynthesis as well as a metabolite of phospholipid. The molecular species of PA were analyzed after convertion to the Phostag® adduct. The derivative gave a monocationic complex $[PA^{2-}ZnL^{3+}]^{+}$, and thus the molecular species were detected in positive mode without no production of other cation adducts (*16*). By using single-isotope zinc, ^{68}Zn in the present study, the molecular species of PA can easily be determined with a small quantity of sample. The main molecular species of PA in bonito muscle was 1-stearoyl-2-docosahexaenoyl PA. 1-Palmitoyl-2-oleoyl PA, which was not formed by the transacylase using endogenous bonito muscle LPC, was also abundant, but the 1,2-didocosahexaenoyl species, which was formed abundantly by the transacylase, was scarcely detected among PA species (Fig. 1A). Together, 2-docosahexaenoyl LPC, which is formed by the PLA_1 hydrolysis of 2-docosahexaenoyl species of PC, such as 1-palmitoyl-2-docosahexaenoyl PC, would serve as a substrate for the LPC/transacylase, and 1,2-didocosahexaenoyl PC would be formed. As far as the fatty acid preference for hydrolysis by the PLA_1, the hydrolysis of 1,2-didocosahexaenoyl PC was one-fourth that of 1-palmitoyl-2-docosahexaenoyl PC (*3*). These joint actions of the PLA_1 and LPC/transacylase would be a rea-

son why the 1,2-didocosahexaenoyl species was abundant in bonito muscle PC (Figs. 1B and 2A). In support of this, the 1,2-didocosahexaenoyl species was not abundant in PE (Fig. 1C), of which the lyso form was not preferred for the transacylation (Table 1).

1,2-Dipolyunsaturated fatty acid species are reported to be most effective for rapid membrane flip-flop (*28*) and phase separation from a lipid raft (*29*). What is the function or role of PC molecular species having highly unsaturated fatty acid at both *sn*-1 and *sn*-2 positions, such as 1,2-diarachidonoyl PC in testis (*23*), 1,2-dieicosapentaenoyl PC in *C. elegans* (*30*), and 1,2-didocosahexaenoyl PC in bonito muscle? This question remains to be solved.

Because the lysophospholipase-transacylase from bonito muscle catalyzes the transacylation of a fatty acyl moiety from one lysophosphatidylcholine to another lysophosphatidylcholine without significant production of free fatty acid, and because lysophosphatidylethanolamine is not the preferred substrate for the transacylation, the term lysophosphatidylcholine/transacylase is applied to this enzyme. This study was partially supported by a grant from Sugiyama Chemical & Industrial Laboratory (2002).

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