Purification and Characterization of 1-O-Acylceramide Synthase, a Novel Phospholipase A₂ with Transacylase Activity*

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A novel pathway for ceramide metabolism, 1-O-acylceramide formation, was previously reported (Abe, A., Shayman, J. A., and Radin, N. S. (1996) J. Biol. Chem. 271, 14383–14389). In this pathway a fatty acid in the sn-2 position of phosphatidylethanolamine or phosphatidylcholine is transferred to the 1-hydroxyl position of ceramide. An enzyme that catalyzes the esterification of N-acetylsphingosine was purified from the postmitochondrial supernatant of calf brain through consecutive steps, including ammonium sulfate fractionation, DEAE-Sephaloc, phenyl-Sepharose, S-Sepharose, Sephadex G-75, concanavalin A-agarose, and heparin-Sepharose chromatography. The molecular mass of the enzyme was determined to be 40 kDa by gel filtration on Sephadex G-75. The enzyme bound to concanavalin A-agarose column was eluted with the buffer containing 500 mM α-methyl-D-man-nopyranoside. Further purification by heparin-Sepharose chromatography resulted in separation of two peaks of enzyme activity. Coincidence between the transacylase activity and a stained protein of a molecular mass of 40 kDa was observed, as determined by SDS-polyacrylamide gel electrophoresis and recovery after separation over an acidic native gel. The second peak of activity from the heparin-Sepharose chromatography represented a purification of 193,000-fold. These results are consistent with the enzyme being a glycoprotein of a molecular mass of about 40 kDa with a single polypeptide chain. The purified enzyme had a pH optimum at pH 4.5. The divalent cations Ca²⁺ and Mg²⁺ enhanced but were not essential for the transacylase activity. Neither activation nor inactivation of the enzyme activity was observed in the presence of 2 mM ATP or 2 mM dithiothreitol. Preincubation of the enzyme with 1 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, or 3.1 μM bromoemol lactone, a potent inhibitor of cytosolic Ca²⁺-independent phospholipase A₂, had no significant effect on the enzyme activity. The enzyme activity was completely abolished in the presence of greater than 773 μM Triton X-100. Partial inhibition of the enzyme activity was observed in the presence of 10–100 μM heparin. In the absence of N-acetylsphingosine, the enzyme acted as a phospholipase A₂. These results strongly suggest that 1-O-acylceramide synthase is both a transacylase and a novel phospholipase A₂.

Recently ceramide has been identified as a candidate lipid mediator regulating cell proliferation, growth, differentiation, development, and apoptosis (1, 2). In many studies, proof of concept for a biological role of ceramide has relied on the use of N-acetylsphingosine (NAS), 1 a truncated and membrane-permeable homologue of endogenous ceramides. Reliance on NAS is problematic since this compound is subject to metabolism when exogenously applied to cultured cells. We observed that MDCK cells and NIH 3T3 cells actively metabolized and converted NAS to other sphingolipids and resulted in a considerable increase in levels of sphingosine, natural (long chain) ceramide, and glucosylceramide (3–5).

Previously reported work on the metabolism of NAS in MDCK cell homogenates revealed the formation of a unique, alkali-labile lipid, 1-O-acylcereamide (5). An enzyme activity that catalyzed esterification of a hydroxyl group at C1 position of NAS was described. The enzyme displayed an acidic pH optimum and was mainly recovered in a soluble fraction after centrifugation at 100,000 × g. In the presence of NAS and using liposomes consisting of phosphatidylcholine, [2-¹⁴]C]arachidonylphosphatidylethanolamine and sulfate, the enzyme catalyzed both the transacylation of NAS and the release of arachidonic acid from phosphatidylethanolamine. In the absence of NAS, the release of arachidonic acid was still observed. The enzyme predominantly catalyzes the deacylation or transacylation of both phosphatidylethanolamine and phosphatidylcholine. Additionally, Ca²⁺ was not required for the enzyme activity. This enzyme appeared to be a novel Ca²⁺-independent PLA₂. In the present paper, we report the purification and characterization of this novel acid phospholipase A₂ with transacylase activity from calf brain.

EXPERIMENTAL PROCEDURES

Materials

The reagents and their sources were 1-O-1-acyl-2-[¹⁴C]arachidonylphosphatidylethanolamine (53 mCi/mmol) from Amersham Pharmacia Biotech; [⁹⁹mTc]acetic anhydride (50–100 mCi/mmol) from American Radiolabeled Chemicals; Ecolume scintillation fluid from ICN; N-ethylmaleimide, Triton X-100, phosphatidylethanolamine (PE) from bovine brain, dicetylphosphate, d-erythro-sphingosine, heparin, phenylmethyisulfonyl fluoride (PMSF), α-methyl-β-mannopyranoside, and S-Sepharose from Sigma; dioleoylphosphatidylcholine (DOPC) from Avanti; m-dithiotheritol (DTT) and nonadecyltetraenyl trifluoroacetoxymethyl ketone (AACOCF₃) from Calbiochem; (E)-6-bromomethylene tetrahydro-3-(1-naphthalenyl)-(2H)-pyran-2-one (BEL) from Cayman Chemical. N-[⁹⁹mTc]Acetylsphingosine ([⁹⁹mTc]NAS) was prepared by the method of

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1 The abbreviations used are: NAS, N-acetylsphingosine; AACOCF₃, nonadecyltetraenyl trifluoroacetoxymethyl ketone; BEL, (E)-6-bromomethylene tetrahydro-3-(1-naphthalenyl)-(2H)-pyran-2-one; PLA₂, phospholipase A₂; cPLA₂ and iPLA₂, cytosolic and calcium-independent PLA₂, respectively; DOPC, dioleoylphosphatidylcholine; DTT, m-dithiother-itol; MDCK, Madin-Darby canine kidney; NEM, N-ethylmaleimide; PMSF, phenylmethyisulfonyl fluoride; PE, phosphatidylethanolamine; Con A, concanavalin A; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.
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Pheny1-Sepharose Chromatography (Step 4)—The unbound fraction was adjusted to 3 mM NaCl and then applied to a column (2.5 x 20 cm) of phenyl-Sepharose preequilibrated with buffer B containing 3 mM NaCl (buffer C) at a flow rate of 80 ml/h. Unbound material was eluted with 1000 ml of buffer C. Additionally the column was rinsed by 450 ml of buffer B containing 0.15 mM NaCl (buffer D) and then by 400 ml of buffer B containing 0.15 mM NaCl (buffer D). A small portion of the enzyme activity was released from the column during rinsing by buffer D. Most enzyme activity was eluted by 400 ml of buffer D containing 50% (v/v) ethylene glycol (buffer E) and kept at −80 °C.

The same procedure, steps 1−4, was separately carried out 4 times. The 4th fraction kept at −80 °C after the phenyl-Sepharose chromatography step was combined and dialyzed against 10 mM sodium phosphate (pH 6.0), 1 mM EDTA (buffer F).

S-Sepharose Chromatography (Step 5)—The dialysate was applied to a column (1.4 x 20 cm) of S-Sepharose preequilibrated with buffer F at a flow rate of 40 ml/h. Unbound protein was eluted by buffer F. The bound protein was eluted by a 450-ml linear gradient of NaCl from 0–0.3 mM NaCl in buffer F. The enzyme activity was found in fractions between 60 and 130 mM NaCl as two peaks. Each peak was separately collected and named “S-Sepharose fast” and “S-Sepharose slow” by following the order of elution.

Sephadex G-75 Chromatography (Step 6)—Both fractions, S-Sepharose fast and S-Sepharose slow, were concentrated by Centricon-10 (Amicon) and then applied to a column (2.6 x 90 cm) of Sephadox G-75 preequilibrated with 0.15 mM NaCl, 25 mM Tris-HCl (pH 7.4), 1 mM EDTA (buffer D) at a flow rate of 40 ml/h. Protein was eluted with buffer D. For S-Sepharose slow, a peak of the enzyme activity appeared at Ve/Vo = 1.41. For S-Sepharose fast, two small peaks of the enzyme activity were observed at Ve/Vo = 1.12 and 1.41. The active fractions obtained from S-Sepharose slow were collected and used for further purification.

Concanavalin A-Agarose Chromatography (Step 7)—The fraction obtained in Step 6 was adjusted to 1 mM CaCl2 and 1 mM MnCl2 and applied to a column (0.85 x 1.8 cm) of Con A-agarose preequilibrated in 500 mM NaCl, 25 mM Tris-HCl (pH 7.4), 1 mM CaCl2, and 1 mM MnCl2 (buffer G) at a flow rate 1.5 ml/h. Unbound protein was eluted by 10 ml of buffer G. The column was rinsed by buffer G including 10 mM α-methyl-n-mannopyranoside. During the rinse, a small portion of the enzyme activity was released from the column. Most enzyme activity was eluted by 10 ml of buffer G including 500 mM α-methyl-n-mannopyranoside (buffer H) and possessed a very high specific activity in the transacylase assay. The active fraction eluted with buffer H was collected, adjusted to 77.3 μM of Triton X-100, and then dialyzed against 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 77.3 μM (w/v) Triton X-100 (buffer I).

Heparin-Sepharose Chromatography (Step 8)—The dialysate was applied to a column of heparin-Sepharose (HTrap heparin, 1 mL; Amersham) preequilibrated in buffer I at a flow rate of 5 ml/h. Unbound protein was eluted by 15 ml of buffer I. Then the column was rinsed by 15 ml of buffer B containing 77.3 μM Triton X-100 (buffer B′). The bound protein was eluted by a 60-ml linear gradient of NaCl from 0 to 0.15 M in buffer B′.

RESULTS

Purification of Transacylase from Calf Brain

Postmitochondrial Supernatant (Step 1)—Fresh calf brains were stored at −80 °C as thin blocks after removal of pia mater. Frozen brains (600 g) were partly allowed to thaw in the cold room overnight and then cut in small pieces, after which all manipulations were performed at 4 °C. A 30% homogenate in 10 mM Tris-HCl (pH 7.4 at room temperature), 1 mM EDTA, 0.5 mM PMSF (buffer A) was prepared by a Polytron for 1 min at 20,000 rpm. The mixture was centrifuged at 14,000 x g for 30 min. The pellet was reextracted as described above.

Ammonium Sulfate Precipitation (Step 2)—Ammonium sulfate was added to the postmitochondrial supernatant to 20% saturation (16 g/100 ml, pH 7.4 at room temperature), 1 mM EDTA (buffer A). The protein was adjusted by centrifugation at 14,000 x g for 30 min. The supernatant was adjusted to 70% saturation with ammonium sulfate (31.2 g/100 ml) to precipitate the enzyme. After standing overnight, the clear supernatant above the precipitate was siphoned off. The precipitate was collected by centrifugation at 10,000 x g for 30 min, dissolved in 60 ml of buffer A, and dialyzed for 2 days against 5 times 4 liters of 50 mM Tris-HCl (pH 7.4, at room temperature), 1 mM EDTA (buffer B). The protein was adjusted to less than 20 mg/ml with buffer B and centrifuged at 100,000 x g for 1 h.

DEAE-Sephalcel Chromatography (Step 3)—The supernatant was applied to a column (5 x 22 cm) of DEAE-Sephalcel preequilibrated with buffer B at a flow rate of 100 ml/h. Unbound protein was eluted with 2 liters of buffer B. The enzyme activity was recovered in the unbound fraction.

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Table I summarizes the various steps of the protocol developed for transacylase purification. An eight-step procedure was employed, and the transacylase activity was monitored by the acylation of NAS throughout the purification.

In the present study, calf brain was chosen as a starting material. The post-mitochondrial supernatant was precipitated by 20–70% ammonium sulfate. After dialyzing the suspension of the precipitates, the 100,000 g supernatant of the dialysate was applied to the DEAE-Sephalcel column, and most of the enzyme activity was recovered in the unbound fraction. The recovery was improved after the DEAE-Sephalcel column. This step gave rise to about 19-fold purification. The unbound fraction was adjusted to 3 mM NaCl and applied to the phenyl-Sephalcel column. The enzyme activity was recovered by the buffer containing 50% ethylene glycol (Fig. 1). This step gave rise to an additional 7.6-fold purification.

In the next S-Sephalcel chromatography, the enzyme activity was eluted in fractions between 60 and 130 mM NaCl as two...
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TABLE I

| Step                        | Volume | Protein | Specific activity | Recovery | Purification factor |
|-----------------------------|--------|---------|-------------------|----------|---------------------|
| 12,000 g of supernatant     | 10,850 | 54,009  | 0.130$^a$        | 100      | 1                   |
| 20–70% saturated ammonium   | 2326   | 23,413  | 0.159             | 52.4     | 1.22                |
| DEAE-Sepharose (unbound)    | 7920   | 3036    | 2.5               | 103      | 19.2                |
| Phenyl-Sepharose (50%       | 780    | 250     | 18.9              | 67.2     | 145                 |
| ethylene glycol)            |        |         |                   |          |                     |
| S-Sepharose (fast)          | 95     | 81.1    | 15.8              | 20.6     | 122                 |
| Sephadex G-75               | 31     | 7.41    | 50.8              | 5.36     | 391                 |
| S-Sepharose (slow)          | 86     | 26.4    | 77.8              | 29.2     | 598                 |
| Sephadex G-75$^b$           | 37     | 9.21    | 96.7              | 12.7     | 744                 |
| Con A-agarose               | 17.6   | 0.0372  | 5.325             | 2.82     | 40,962              |
| Heparin-Sepharose           | 3.7    | 0.0045  | 25.149            | 1.62     | 193,450             |

$^a$ Transacylase activity was measured as described under "Experimental Procedures" and as originally described (5).

$^b$ In order to obtain the enzyme activity, the postmitochondrial supernatant was centrifuged for 60 min at 100,000 × g, and the specific activity was determined using the membrane-free supernatant.

$^c$ Fraction S-Sepharose fast was used.

$^d$ Fraction S-Sepharose slow was used. The active fractions from this Sephadex G-75 chromatography step was pursued for further purification.

Fig. 1. Phenyl-Sepharose CL-4B column chromatography.

The active fraction obtained from the DEAE-Sepharose chromatography step was adjusted to 5 mM NaCl and applied to the phenyl-Sepharose CL-4B column. Arrows at Buffer D and Buffer E indicate the point at which elution buffers were changed to 0.15 mM NaCl, 25 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 50% ethylene glycol, 0.15 mM NaCl, 25 mM Tris-HCl (pH 7.4), 1 mM EDTA, respectively. The column was eluted as described under "Experimental Procedures." 6.6 ml fractions and 5.5 ml fractions were collected in tubes number 1–57 and 76–120, respectively. Thirty-μl aliquots were assayed for the transacylase activity (■). Protein was measured as absorbance at 280 nm (○).

Fig. 2. S-Sepharose column chromatography.

The active fraction from the phenyl-Sepharose chromatography step (fractions 76–105) was dialyzed against 10 mM sodium phosphate (pH 6.0), 1 mM EDTA (buffer F). The dialysate was applied to the S-Sepharose column and eluted as described under "Experimental Procedures." Six-ml fractions were collected in tubes. Fifteen-μl aliquots were assayed for the transacylase activity (■). (○) and (- - -) denote absorbance at 280 nm and NaCl concentration, respectively.

There was no enrichment in the purification of the S-Sepharose fast fraction. On the other hand the S-Sepharose slow fraction was purified 4.1-fold.

Before step 6 (Sephadex G-75 gel filtration), both S-Sepharose fast and S-Sepharose slow fractions were concentrated using Centriprep-10 without loss of enzyme activity. Only a small increase in purity was produced by gel filtration. For S-Sepharose slow, a peak of the enzyme activity appeared at Ve/Vo = 1.41 (Fig. 3B). On the other hand, for S-Sepharose fast two small peaks of the enzyme activity were observed at Ve/Vo = 1.12 and 1.41 (Fig. 3A). The values of Ve/Vo, 1.12 and 1.41, corresponded to molecular masses of 77.5 kDa and 38.6 kDa, respectively. The active fractions shown in the Fig. 3B were collected and used for further purification.

Most proteins passed through the Con A agarose column (Fig. 4). Buffer containing 500 mM α-methyl-d-mannopyranoside was required to elute the enzyme (Fig. 4). This step gave rise to an additional 55-fold purification. Further recovery of the enzyme was accomplished by maintaining the column in buffer H overnight without elution and then rinsing with the same buffer. Because the protein concentration in the active fraction was extremely low, the protein solution was stabilized with 77.3 μM Triton X-100. At this stage, the purified transacylase was separated by acidic native gel electrophoresis. The extraction of gel slices and assay of transacylase activity corresponded to a protein of a molecular mass 40 kDa (Fig. 5).

The active fraction obtained from the Con A column was applied to the heparin-Sepharose column and eluted by a linear gradient of 0–150 mM NaCl. No Con A and Con A fragments were contaminated in the sample after heparin-Sepharose chromatography. The enzyme activity appeared as two peaks between 20 and 50 mM NaCl (Fig. 6). In this single experiment the elution profile of the enzyme activity corresponded to that of protein of a molecular mass 40 kDa, estimated by SDS-PAGE. The lack of a protein band in lane 30 was due to the accidental loss of deoxycholate-trichloroacetic acid precipitate during aspiration. The highly purified enzyme found in the second peak was purified 193,000-fold. The protein appeared as a single band by SDS-PAGE and was used for further characterization of the enzyme. The yield from 10 calf brains (2,400 g) was 4.5 μg with a specific activity of 25 μmol/min/mg of protein. The overall recovery of the activity was 1.6%. Transacylase activity was always accompanied with phospholipase A activity throughout the purification steps as determined by thin layer chromatography and charring (for example, Fig. 5).
The enzyme activity was totally abolished at pH 5–6.5 (5). The enzyme activity was slightly inhibited in the presence of greater than 77.3 μM Triton X-100 if the final concentration of Triton X-100 in the assay system was reduced less than 773 nM. However, the transacylase activity was strongly affected in the presence of higher concentrations of Triton X-100. The activity completely disappeared in the presence of greater than 773 μM Triton X-100 (Fig. 9).

In a preliminary test, it was found that the enzyme activity in the active fraction of Sephadex G-75 eluate (from S-Sepharose slow) was partially inhibited by 100 μg/ml heparin. As already shown in Fig. 6, the enzyme was bound to heparin-Sepharose resin. Fifty percent inhibition of the enzyme activity was observed in the presence of 10–100 μg/ml heparin (Table II).

**Dual Enzyme Activity, Transacylase and Phospholipase A₂—**As noted above, the transacylase was always accompanied with phospholipase A activity. To confirm the existence of both transacylase and phospholipase A activities, 1-α-1-acyl-2-[1-14C]arachidonoyl-PE was employed as an acyl donor. The enzyme acted as not only a transacylase but also as a PLA₂ in the presence of NAS as an acceptor. Interestingly, the total activity of the transacylase and PLA₂ in the presence of NAS was exactly equal to PLA₂ activity in the absence of NAS as an acceptor. Interestingly, the total activity of the transacylase and PLA₂ in the presence of NAS was exactly equal to PLA₂ activity in the absence of NAS. Only 7% of total radiolabeled products was 2-[1-14C]arachidonoyllysophosphatidylethanolamine after 30 min incubation in the presence or absence of NAS. These data are consistent with a high degree of specificity for the enzyme in using the sn-2 fatty acid of PE.

**DISCUSSION**

Our previous study demonstrated that the transacylase activity of brain is higher than that of other tissues (5). Therefore...
brain was chosen as a starting material. In a preliminary study, it was confirmed that the transacylase activity is efficiently released by sonication from MDCK cell homogenate prepared in isotonic buffer, consistent with its localization in an intracellular compartment. In the present study, fresh calf brains were frozen once before use. The thawed tissues were homogenized with a hypotonic buffer using a Polytron. This procedure presumably lysed intracellular compartments efficiently and resulted in an increased release of the enzyme. However, a very low recovery (20–30%) of enzyme activity was initially obtained after ammonium sulfate fractionation. Recovery was markedly improved by diluting the dialysate before the 100,000 g centrifugation. A high protein concentration might have caused a nonspecific coprecipitation of the enzyme with insoluble materials during ultracentrifugation. The observed rebound of enzyme activity after DEAE-Sephacel chromatography might have been due to separation of the enzyme from an endogenous inhibitor.

The concanavalin A-agarose chromatography step was one of the most successful purification steps. The enzyme was tightly bound to concanavalin A-agarose resin and specifically released from the resin with 500 mM $\alpha$-methyl-d-mannopyranoside (Fig. 4). At this stage, a major band of molecular mass...
about 40 kDa and some minor bands of that between 43 and 60 kDa were observed in the active fractions from the concanavalin A column by SDS-PAGE (Fig. 5). Further purification by the heparin-Sepharose column showed an exact coincidence between the transacylase activity and a stained protein of a molecular mass of about 40 kDa as determined by SDS-PAGE (Fig. 6), consistent with the molecular mass assignment by Sephadex G-75 chromatography (Fig. 3). These data are consistent with the purified transacylase being a 40-kDa oligomannose-containing glycoprotein with a single polypeptide chain. This enzyme has a pH optimum of 4.5. Although suggestive of a lysosomal origin for the transacylase, the enzyme activity was still observed at pH 5–6.5 (Fig. 6). Thus the enzyme may function outside the acidic environment of lysosomes.

Secreted PLA₂s, group I, II, and III, contain 5–8 disulfide bonds essential for the enzyme activity (13, 14). Reducing these disulfide bonds by thiol reagents results in inhibition of the lipase activity. The calf brain transacylase activity was insensitive to DTT when preincubated at pH 7.4 in Tris buffer or when added directly to the assay mixture (Table II). Using a partially purified enzyme (step 7), it was found that the enzyme was tightly bound to organomercurial-agarose resin (Affi-Gel 501; Bio-Rad) and eluted by 200 mM 2-mercaptoethanol (data not shown). This indicates that the enzyme has free thiol group(s) exposed on its molecular surface. Pretreatment of the purified enzyme with NEM had no effect on the enzyme activity (Table II). These results suggest that the free thiol group(s) of the enzyme that is thiol reagent-sensitive is neither functionally important nor found in the catalytic site.

Recently human cPLA₂ was found to have a transacylase activity that is CoA-independent, raising the possibility that cPLA₂ forms an acyl-enzyme intermediate (15). Site-directed mutagenesis of cPLA₂ demonstrated that the serine 228 essential for the catalytic activity of the 85-kDa cPLA₂ may be the

| Additions         | Percent of control activity |
|-------------------|----------------------------|
| None              | 100 ± 5                    |
| EDTA (5 mM)       | 77 ± 2                     |
| CaCl₂ (1 mM)      | 148 ± 4                    |
| CaCl₂ (10 mM)     | 127 ± 7                    |
| MgCl₂ (1 mM)      | 132 ± 11                   |
| MgCl₂ (10 mM)     | 143 ± 6                    |
| ATP (2 mM)        | 82 ± 3                     |
| DTT (10 mM)       | 94 ± 6                     |
| NEM (1 mM)        | 95 ± 1                     |
| PMSF (1 mM)       | 80 ± 4                     |
| BEL (3.1 μM)      | 86 ± 14                    |
| Heparin (10 μg/ml)| 52 ± 3                     |
| Heparin (100 μg/ml)| 50 ± 1                    |

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**Table II**

The effect of inhibitors and activators on purified transacylase

The control incubation consisted of 40 mM sodium acetate (pH 4.5), 10 μM [3H]NAS, 10 μg/ml BSA, DOPC/PE/dicetylphosphate liposomes and 6 ng/ml enzyme. The enzyme activity of the control was 20 μmol/min/mg of protein. In the experiments with NEM, DTT, or PMSF, the enzyme was preincubated for 40 min at 0 °C with the compounds in Tris buffer at pH 7.4. In the experiment with BEL, the enzyme was preincubated for 6 min at 25 °C with 3.1 μM BEL. Values are mean ± S.D. for n = 3 except for ATP and NEM (n = 2).

**Fig. 8. Effect of AACOCF₃ on transacylase activity.** The reaction mixture contained 38.5 mM sodium citrate (pH 4.5), DOPC/PE/dicetylphosphate (7/3/1, 128 μM phospholipid) liposomes, 10 μg/ml BSA, 10 μM [3H]NAS, 6 ng/ml purified transacylase in the absence or presence of 28 μM AACOCF₃. Incubation was carried out at 37 °C. Values represent the mean ± S.D. (n = 3).

**Fig. 9. Effect of Triton X-100 on transacylase activity.** The reaction mixture contained 38.5 mM sodium citrate (pH 4.5), DOPC/PE/dicetylphosphate (7/3/1, 128 μM phospholipid) liposomes, 10 μg/ml BSA, 10 μM [3H]NAS, 3.07 μg/ml active fraction from the Sephadex G-75 column (Fig. 5B) in the absence or presence of Triton X-100. Incubation was for 10 min at 37 °C. Values represent the mean ± S.D. (n = 3).

**Fig. 10. Dual enzyme activity.** The reaction was carried out with 38.5 mM sodium citrate (pH 4.5), DOPC/PE/dicetylphosphate (7/3/1, 128 μM phospholipid) liposomes containing L-a-1-acyl-2-[1-14C]arachidonyl-PE (130,000 cpm), 10 μg/ml BSA, 6 ng/ml purified transacylase in the absence or presence of 10 μM N-acetysphingosine. The incubation was carried out at 37 °C. The formation of O-acyl-NAS and the release of arachidonic acid in the absence of enzyme was used as a control at each point and subtracted as a blank from the total activity.
active site nucleophile. (17). Additionally, it was reported that AAcOCF₃ is both a slow and tight binding inhibitor of cPLA₂ that may form a hemiketal enzyme-inhibitor complex (16, 18). The reaction mechanism of cPLA₂ thus might be similar to that of serine protease, esterase, or lipase (15–18). In general, these types of enzymes are also sensitive to PMSF and diisopropylfluoride. In the present study, neither 1 mM PMSF nor 28 μM AAcOCF₃ significantly inhibited the activity of the purified transacylase (Table II and Fig. 8). The action of these inhibitors on the enzyme in 100,000 × g supernatant obtained from MDCK cells homogenate was also not significant (5). These results suggest that neither the calf brain transacylase nor MDCK cell transacylase has an essential serine residue for the transacylase reaction.

Although the purified transacylase did not require Ca²⁺ or Mg²⁺ for activity, these cations enhanced the activity (Table II). In the previous study, it was observed that 1 mM Ca²⁺ slightly enhanced the transacylase activity in the 100,000 × g supernatant obtained from MDCK cells homogenate (5). It is known that divalent cations affect the physical state of lipid assembly (12). The enhancement of the enzyme activity is thought to be due to formation of ionic bridges by these divalent cations on liposomes, thereby modulating molecular packing and availability of substrate.

Recently, it has been reported that myocardial cytosolic iPLA₂ and macrophage cytosolic iPLA₂ interact with ATP, acting on the enzyme as an activator or a stabilizer (10, 11). The purified calf brain transacylase was not activated in the presence of ATP (Table II). It has also been reported that BEL is a potent covalently bound inhibitor of these iPLA₂s but not other PLA₂s (secreted Ca²⁺-dependent PLA₂ or cytosolic Ca²⁺-dependent PLA₂) (19, 20). Myocardial iPLA₂ activity was completely abolished by pre-incubation of the iPLA₂ with 1 μM BEL (19), and half-maximal inhibition of macrophage iPLA₂ activity was observed at 60 nM after a 5 min preincubation (20). Preincubation of the calf brain transacylase with 3 μM BEL had no significant effect on the enzyme activity (Table II). These results suggest that a functional group sensitive to BEL inhibitors does not exist around the active site and/or lipid recognition site of the calf brain transacylase.

Bartolf and Franson (22) reported that a lysosomal iPLA₂ from bovine adrenal medulla was stabilized by adding Triton X-100, although the lipase activity was reduced in a dose-dependent manner at higher Triton X-100 concentrations (22). A similar effect was observed on the purified calf brain transacylase, where activity was completely abolished in the presence of more than 773 μM Triton X-100 (Fig. 9). On the contrary, iPLA₂ (39 kDa) partially purified from bovine brain has the optimal concentration of Triton X-100 at 773–1546 μM, suggesting that the transacylase is distinct from the 39-kDa iPLA₂ (27).

Using iPLA₂ (39 kDa) partially purified from bovine brain, Yang et al. (21) reported that heparin at either 100 or 200 μg/ml produced 25% inhibition of the iPLA₂ activity. The calf brain transacylase was bound to heparin-Sepharose resin under a low ionic condition and eluted by a low concentration of NaCl consistent with weak ionic binding (Fig. 6). In the present study, 50% inhibition of the transacylase activity occurred at 10–100 μg/ml heparin (Table II). The interaction between the enzyme and heparin might be very weak in the assay system because of the ionic strength. Alternatively, heparin is thought to interact with liposomes and to change the surface properties and the molecular packing of liposomes. As a result, an apparent activity of the enzyme might be partially suppressed.

iPLA₂s have been found in many mammalian tissues and cells (23), and some successful purification of iPLA₂s has been reported, including canine myocardium (10), P388D₁ macrophages (11), and rat lung (24), brain (25), and kidney (26). Some biochemical properties of the calf brain transacylase, including insensitivity to DTT and Ca²⁺ independence for enzyme activity, are found among iPLA₂s. However, many of iPLA₂s have a neutral pH optimum. For example, although two kinds of cytosolic iPLA₂, 110 kDa and 39 kDa, were partially purified from bovine brain, the optimal pH values of 110-kDa and 39-kDa enzymes were 7.4 and 8.0, respectively (27). The purified rat kidney soluble iPLA₂ has a molecular mass of 28 kDa and a neutral pH optimum (26). Although a soluble acidic pH optimum iPLA₂ was purified from rat brain by a protocol similar to ours, the enzyme displayed a molecular mass of 58 kDa and was highly phosphatidic acid-selective (25). Recently Wang et al. (24) purified an acidic-pH optimum iPLA₂ from rat lung. This enzyme displayed some properties similar to the calf brain transacylase, including insensitivity to 2-mercaptoethanol, ATP, AAcOCF₃, and the binding ability to heparin-Sepharose resin. However, the molecular mass of the lung iPLA₂ was 15 kDa (24). A soluble, lysosomal iPLA₂ partially purified from bovine adrenal medulla showed a basic pI, Triton X-100 inhibition, and binding to concanavalin A-agarose resin (22). These profiles are common to the calf brain transacylase. This enzyme, however, gave a molecular mass of 30.6 kDa.

More recently, a calcium-independent lysosomal PLA₂ has been cloned. This enzyme displayed a similar pharmacologic profile to the 15-kDa protein but has a molecular mass of 25.8 kDa (28).

In the previous study, it was strongly suggested that MDCK cell transacylase has dual enzyme activity, i.e. transacylase and PLA₂ (5). In the present study, both transacylase and phospholipase A activities were observed in the purified protein. The assay in which DOPC(α-1-acyl-2-[1-14C]arachidonoyl-PE/dicyethylphosphate liposomes was used as an acyl donor demonstrated that the calf brain transacylase possesses dual enzyme functions, viz. transacylase and phospholipase A activities (Fig. 10). The enzyme also produced 2-[14C]arachidonoyl-lyso-PE but only as a small fraction of the total radioactive products. This supports the view that the phospholipase activity of the transacylase has sn-2 fatty acid specificity. These data are consistent with the interpretation that 1-O-acetyl-lysoamide synthase is a novel Ca²⁺-independent PLA₂.

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