Purification and Characterization from Rat Kidney Membranes of a Novel Platelet-activating Factor (PAF)-dependent Transacetylase That Catalyzes the Hydrolysis of PAF, Formation of PAF Analogs, and C₂-ceramide*

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We have previously identified two enzyme activities that transfer the acetyl group from platelet-activating factor (PAF) in a CoA-independent manner to lysosphasmalogen or sphingosine in HL-60 cells, endothelial cells, and a variety of rat tissues. These were termed as PAF:lysosphasmalogen (lysosphospholipid) transacylase and PAF:sphingosine transacylase, respectively. In the present study, we have solubilized and purified this PAF-dependent transacylase 13,700-fold from rat kidney membranes (mitochondrial plus microsomal membranes) based on the PAF:lysosphasmalogen transacylase activity. The mitochondria and microsomes were prepared and washed three times, then solubilized with 0.04% Tween 20 at a detergent/protein (w/w) ratio of 0.1. The solubilized fractions from mitochondria and microsomes were combined and subjected to sequential column chromatographies on DEAE-Sepharose, hydroxyapatite, phenyl-Sepharose, and chromatofocusing. The enzyme was further purified by native-polyacrylamide gel electrophoresis (PAGE) and affinity gel matrix in which the competitive inhibitor of the enzyme, 1-O-hexadecyl-2-N-methylcarbamyl-sn-glycero-3-phosphoethanolamine was covalently attached to the CH-Sepharose. On SDS-PAGE, the purified enzyme showed a single homogeneous band with an apparent molecular mass of 40 kDa. The purified enzyme catalyzed transacyltransferation of the acetyl group not only from PAF to lysosphasmalogen forming plasmalogens analogs of PAF, but also to sphingosine producing N-acetylsphingosine (C₂-ceramide). In addition, this enzyme acted as a PAF-acetylhydrolase in the absence of lipid acceptor molecules. These results suggest that PAF-dependent transacyltransferase is an enzyme that modifies the cellular functions of PAF through generation of other diverse lipid mediators.

Platelet-activating factor (PAF),¹ 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (alkylacetyl-GPC), is a potent lipid mediator with a wide variety of biological activities related to physiological and pathological phenomena (1, 2). PAF is produced by either de novo or remodeling pathway (1). In addition, we found the PAF could also be metabolized by a novel pathway catalyzed by membrane-associated transacyltransferase that transfers the acetyl group of PAF to lysosphasmalogen in HL 60 cells (3). This enzyme is CoA-independent and transfers the acetyl group from PAF to a variety of lysosphospholipids acceptors in the order of radyl-GPC > radyl-glycerophosphoethanolamine (GPE) > acyl-glycerophosphoserine > acyl-glycerophosphoinositol > acyl-glycerophosphate > alkyl-glycerophosphate > fatty alcohol, whereas alkylglycerol, acylglycerol, or cholesterol are inactive as acceptors. This PAF-dependent transacyltransferase is participated in the biosynthesis of acyl analog of PAF (4), which is the predominant molecular species of PAF in hematopoietic cells including endothelial cells, mast cells, and basophils, etc. (5). In endothelial cells, PAF-dependent transacyltransferase activity is regulated by phosphorylation/dephosphorylation (4).

Recently, we have demonstrated that a similar PAF-dependent transacyltransferase transfers the acetyl group of PAF to sphingosine in HL 60 cells (6, 7). This enzyme activity appears to be responsible for the presence of acetylsphingosine (Cᵡ-ceramide) in the biological systems. For instance, Cₓ-ceramide occurred in the micromolar range in undifferentiated and differentiated HL-60 cells (6). This is the concentration range that Cₓ-ceramide has been shown to exert as a second messenger and a lipid mediator by many investigators (8, 9). Since Cₓ-ceramide has many biological activities that differ from PAF and sphingosine, therefore, this enzyme may serve as a modifier for the functions of PAF and sphingosine (6). Both enzyme activities are also found in rat tissues (6). Rat kidney membrane fractions have the highest PAF:sphingosine transacyltransferase activity, while both rat kidneys and lung have the highest PAF:lysosphasmalogen transacyltransferase activity.

To elucidate the relationship of both enzyme activities, we attempted to purify the transacyltransferase from rat kidney membranes, in which the highest enzyme activity toward both lipid acceptors (6, 7). In the present report, we have achieved purification of the transacyltransferase to homogeneity and shown that this enzyme possesses three catalytic activities, namely, PAF-acetylhydrolase, PAF:lysosphospholipid transacylase, and PAF:sphingosine transacylase.

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The abbreviations used are: PAF, platelet-activating factor; alkyl-acetyl-GPC; 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine; GPE, sn-glycero-3-phosphoethanolamine; PAF-AH, platelet-activating factor acetylhydrolase; BSA, bovine serum albumin; DTT, dithiothreitol; Pe-fabloc; p-aminoethylnbenzenesulfonfluoride; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); NEM, N-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; TLC, thin layer chromatography; LCAT, lecithin-cholesterol acyltransferase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid.
Purification of PAF-dependent Transacetylase

EXPERIMENTAL PROCEDURES

Materials—1-O-Hexadecyl-2-acetyl-GPC was obtained from Sigma. 1-O-Hexadecyl-2-N-methylcarbamyl-GPC and C2- ceramide were purchased from Biomol. 1-O-Alkyl-2-acetyl-GPC was from Avanti. Sphingosine was bought from Matreya, Inc. Alkenyllsylo-GPE was a product of NEN Life Science Products. DEAE-Sepharose, phenyl- Sepharose, activated CH-Sepharose, PBE 94, and Polybuffer 74 were from Pharmacia Fine Chemicals (Uppsala, Sweden). Hydroxyapatite was obtained from Bio-Rad. Adult male Sprague-Dawley rats were from Taconic.

Enzyme Assays—PAF:lysoplasmalogen transacylate and PAF: sphingosine transacetylase activities were determined according to our previously described methods (3, 6). The assay system of PAF:lyso- plasmalogen transacylate was consisted of 0.1 ml of 1-O-hexadecyl-2-[3H]acyl-GPC (0.3 µCi), 300 µM lyso- plasmalogen (suspended in 0.1% bovine serum albumin (BSA)-saline), 100 mM Tris-HCl (pH 7.4), 2 mM sodium acetate, and 10 mM EDTA in a total volume of 250 µl. Incubations were carried out at 37 °C for 15 min. The lipids were separated by thin layer chromatography (TLC) using a solvent system of CHCl3/CH3OH/CH3COOH/H2O (50:25:8:4, v/v/v/v), and the radioactivities of the areas corresponding to PAF and alk-1-enylacetyl-GPE were counted using liquid scintillation fluid. The assay system of PAF:sphingosine transacylate contained 15 µM 1-O-hexadecyl-2-[3H]acyl-GPC (1 µCi), 50 µM sphingosine (suspended in equal molar ratio of BSA), 100 mM Tris-HCl (pH 7.4), 2 mM sodium acetate, and 10 mM EDTA in a total volume of 250 µl. Incubations were carried out at 37 °C for 30 min. The lipids were separated by thin layer chromatography (TLC) using a solvent system of CHCl3/CH3OH/CH3COOH/H2O (50:25:8:4, v/v/v/v), and the radioactivities of the areas corresponding to PAF and C2-ceramide were measured. PAF-AH activity was assayed according to the method previously described (11). The assay system of PAF-AH was composed of 20 µM 1-O-hexadecyl-2-[3H]acyl-GPC (0.1 µCi), 1 mM EDTA, and 100 µM potassium phosphate (pH 8.0) in a total volume of 500 µl. Incubations were carried out at 37 °C for 10 min. The reaction was stopped by sequential additions of 1 ml of CHCl3, 1 ml of CH3OH, 0.5 ml of 10% sodium bicarbonate. The upper phase was washed with 1 ml of CHCl3 three times, and the radioactivities in an aliquot of 0.4 ml were determined.

Preparation of Rat Kidney Membranes—The kidneys were dissected out from male rats weighing 150–250 g and homogenized with four volumes of 0.25 m sucrose, 20 mM Tris-HCl (pH 7.4), 1 mM DTT, 1 mM EDTA, and 1 µg/ml leupeptin. The homogenates were centrifuged at 440 × g for 10 min; the supernantant was collected as postnuclear fraction. The postnuclear fraction was centrifuged at 15,000 × g for 10 min to isolate the mitochondrial pellets. The postmitochondrial fractions were centrifuged at 100,000 × g for 1 h to obtain the microsomal fractions. Both mitochondrial and microsomal fractions were washed with the same buffer solution twice, and the washed mitochondria and microsomes were suspended in 20 mM Tris-HCl (pH 7.4), 1 mM DTT, 1 µg/ml leupeptin and 0.02% Tween 20. The column was washed with the same solution, and the enzyme activity was eluted with 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM DTT, 1 µg/ml leupeptin and 0.02% Tween 20.

The active fractions of DEAE-Sepharose were pooled from phenyl-Sepharose and were applied onto a column of 1-O-hexadecyl-2-N-methylcarbamyl-GPC (5 mg, 9.28 µmol) dissolved in 0.5 ml of 0.16 m acetate (pH 5.6) containing 80 mM CaCl2 was combined with 5 mM 1-hexadecyl-2-[3H]acetyl-GPC (10 μCi) and 50 mM 1-hexadecyl-2-[3H]methylcarbamyl-GPE (5 mg, 9.28 µmol) dissolved in 0.5 ml of 0.16 m acetate (pH 5.6) containing 80 mM CaCl2. The reaction product, 1-O-hexadecyl-2-[3H]methylcarbamyl-GPC, was purified by TLC using a solvent system of CHCl3/CH3OH/CH2COOH/H2O (50:25:8:4, v/v/v/v), and the radioactivities of the areas corresponding to CHCl3 were eluted by decreasing pH with Polybuffer 74 (pH 4.0) in 1 mM DTT and 0.02% Tween 20. Five-mI fractions were collected into the tube containing 0.7 ml of 1 mM Tris-HCl (pH 7.4) to neutralize the pH. The enzyme activity was unstable at pH 5.0, and 88% of enzyme activity was lost 16 h thereafter. It was necessary to neutralize the pH of the sample solution soon after the enzyme was eluted from the column. The active fractions from chromatofocusing were concentrated into 4.5 ml by using a small size of hydroxyapatite column (1 × 0.27 cm, 1 ml).

Preparation of Affinity Gel Matrix—1-O-Hexadecyl-2-N-methylcar- bamyl-GPC (5 mg, 9.28 µmol) dissolved in 0.5 ml of 0.16 m acetate (pH 5.6) containing 80 mM CaCl2 was combined with 1 ml of cabbage phospholipase D and 0.5 ml of 20% ethanolamine. The reaction was carried out at room temperature for 16 h (similar to that described in Reference 10). The resulting gel was washed with 50 mM borate (pH 8.0) in CH3OH thoroughly, and the remaining reactive sites were blocked with 1 mM Tris-HCl (pH 8.0) for 16 h at room temperature. Based on results from phosphorus determinations (14), 0.98 µmol of ligand was bound to 1 ml of CH-Sepharose.

Purification of the Enzyme by Affinity Gel Matrix—The active fractions of native-PAGE were combined with 50 µl of affinity gel matrix in a microcentrifuge tube, which was equilibrated with 20 mM Tris-HCl (pH 7.4) containing 0.1 mM NaCl, 1 mM DTT, and 0.02% Tween 20 and mixed gently at 4 °C for 30 min. The gel was washed with the same buffer and followed by the same solution without NaCl. The washing gel was combined with 5 mM alkylacetyl-GPC dissolved in 20 mM Tris-HCl (pH 7.4) containing 0.1 mM DTT, and 0.02% Tween 20 and incubated for 1 h at room temperature. The mixture was centrifuged for 5 min at 10,000 × g, and the supernatant with enzyme activity was transferred into another tube. The tube PAF in this enzyme preparation was removed by hydroxyapatite (0.5 ml) column chromatography. The resulting enzyme solution was dialyzed against 20 mM Tris-HCl (pH 7.4) containing 40% glycerol, 1 mM DTT, and 0.02% Tween 20. The purified preparation was stored at −20 °C, and no significant decrease in enzyme activity was observed at least for 1 month.
SDS-PAGE—SDS-PAGE was carried out according to the method of Laemmli (15) using 10% polyacrylamide gel. The proteins were visualized by silver staining using a silver staining kit for protein (Pharmacia Fine Chemicals).

Sequencing—The sequencing of the protein was carried out at Harvard Microchemistry Facility (Boston, MA) by tandem mass spectrometry and Edman degradation analysis. The purified protein was subjected to electrophoresis on 10% SDS-PAGE gel. The protein band on the gel was visualized by Coomassie Brilliant Blue. The sliced gel was subjected to digestion with trypsin and microsequencing.

Protein Determination—In most instances, protein was measured by a protein assay kit (Bio-Rad) using BSA as the standard. However, when the protein concentrations in the samples were low (e.g., Table III, steps 3–7), a series of one to one (1:1) dilutions of 1 µg/ml BSA (ranging from 31.25 ng to 1 µg per spot) and the samples in duplicates were spotted on a 3MM filter paper. The spots were allowed to air-dry and were stained with 0.25% Coomassie Brilliant Blue R-250 (Bio-Rad), 45% CH3OH, 10% CH3COOH for 30 min. The paper was destained with 45% CH3OH, 10% CH3COOH until the background was nearly white. Then, the protein amount was determined by comparing the intensities of the spots from samples with those from BSA standards. The amount of protein in the final enzyme preparation (Table III, step 8) was estimated by comparing the intensity of the protein band with those of standard proteins after SDS-PAGE and silver staining.

RESULTS

Purification of PAF-dependent Transacylase—An essential step in purifying the membrane enzyme is to first solubilize the enzyme from the membrane. Although there are assortments of detergents (ionic and non-ionic) and chaotropic agents available commercially, the detergent selected will be the one that does not inhibit or inactivate enzyme activity, does not present the enzyme in an aggregated form, and has a high critical micellar concentration. Additionally, the ratio of protein to detergent concentration is important. In preliminary experiments, among the several ionic and non-ionic detergents we tested (such as CHAPSO, octylglucoside, and Triton X-100), Tween 20 concentrations and Tween 20:protein ratios on the solubilization of PAF:lysoplasmalogen transacylase from the total membrane fraction of rat kidneys were measured to ensure proper recovery. Data represent the average of at least duplicate determinations.

| Tween 20 concentration (%) | Tween 20/protein | Activity in supernatant | Protein in supernatant | Purification |
|---------------------------|-----------------|-------------------------|-----------------------|--------------|
| % w/w                     |                 | %                       | %                     | fold         |
| 0.02                      | 0.1             | 41.3                    | 9.3                   | 4.2          |
| 0.02                      | 1.0             | 41.3                    | 19.9                  | 2.1          |
| 0.02                      | 2.0             | 45.4                    | 13.9                  | 3.1          |
| 0.04                      | 0.1             | 37.7                    | 10.6                  | 3.6          |
| 0.05                      | 0.1             | 43.2                    | 9.5                   | 4.5          |

TABLE II

Solubilization of PAF:lysoplasmalogen transacylase from mitochondrial and microsomal fractions of rat kidneys

Four groups of mitochondrial and microsomal fractions were independently prepared from 21 rats as described under “Experimental Procedures.” The solubilization of enzyme was carried out with 0.04% Tween 20 at a detergent/protein (w/w) ratio of 0.1. Values were represented as means ± S.E. (n = 4) per kidney.

| Protein               | Total activity | Specific activity | Recovery |
|-----------------------|----------------|------------------|----------|
|                       | mg             | nmol/min         | nmol/min/mg | %        |
| Mitochondria          | 68.4 ± 1.2     | 142 ± 18.5       | 2.1 ± 0.3  | 100      |
| Microsome             | 48.3 ± 3.2     | 284 ± 13.1       | 6.0 ± 0.4  | 100      |
| First solubilization  | 92 ± 1.0       | 117 ± 3.9        | 14.7 ± 1.2 | 82.3     |
| Second solubilization | 104 ± 0.8      | 11.6 ± 2.2       | 1.2 ± 0.3  | 8.2      |
| First solubilization  | 6.1 ± 0.4      | 143 ± 8.9        | 23.6 ± 1.7 | 50.4     |
| Second solubilization | 5.8 ± 0.6      | 33.7 ± 2.2       | 5.9 ± 0.5  | 11.9     |
was required. The concentration of the ligand in the gel matrix was estimated to be 0.98 mM; therefore, a relatively high concentration of PAF was needed for replacement. These results also indicate that the enzyme specifically recognizes the ligand at the site, which is necessary for the interaction with the substrate. Starting from rat kidney membrane, 13,700-fold purification of the enzyme was achieved with a yield of about 4%. A typical result of the purification procedures is summarized in Table III.

### Purity and Molecular Weight of the Enzyme

Different fractions obtained during the enzyme purification were examined by SDS-PAGE (Fig. 2). The enzyme obtained after the final step of purification showed a single homogeneous band. By comparing the mobility of the enzyme band on SDS-PAGE with molecular size markers, a molecular mass of 40 kDa was deduced for the enzyme (Fig. 3). In addition, the intensity of the band of 40 kDa on SDS-PAGE was correlated with enzyme activity in the individual purification steps including chromatofocusing, native-PAGE, and affinity gel matrix (data not shown), suggesting that the band of 40 kDa is PAF-dependent transacetylase. Although we have attempted to estimate the molecular weight of natural enzyme on gel filtration column of Sephacryl S-200, the enzyme activity was eluted near the void volume due to the formation of mixed micelles with Tween 20 (data not shown).

### Substrate Specificity

A different type of transacetylase that transfers the acetate of PAF to sphingosine forming C2-ceramide was described in HL 60 cells by us (6). The ratios of specific activity toward lysoplasmalogens/sphingosine were 49.7 and 80.8 in crude mitochondrial and microsomal membranes, respectively. In the final purified preparation, it also contains PAF:sphingosine transacetylase activity. Nevertheless, the sphingosine transacetylase activity was more labile during purification and storage than that of lysoplasmalogen transacetylase. Thus, we could not accurately assess the ratio of lysoplasmalogen/sphingosine transacetylase activities. Notwithstanding, these data indicate that a single enzyme can catalyze both kinds of transacetylase activities. The higher ratio toward lysoplasmalogens as the substrate may partly be explained by the fact that, in order to assay PAF:sphingosine transacetylase activity, we had to include equal molar ratio of BSA in the incubations, and BSA severely inhibited sphingosine transacetylase reaction (6). However, incorporation of BSA as the assay medium for sphingosine transacetylase was nec-

### Partial Amino Acid Sequences

The purified protein was digested with trypsin, and five of the peptide fragments were sequenced as described under “Experimental Procedures.” Amino acid sequences of five peptide fragments were:

- GTLDPYEQEVMVR
- AML*AF*L*QK
- LFSSGTR
- IKEGEKEFHVVR
- L*PVSWNGPF*K*

(* indicate that isobaric amino acid residues cannot be unambiguously differentiated in mass spectrometric sequence, but such residues are displayed by alignment with a known or homology to a known, and with reference to enzyme specificity). A search using a protein sequence data bank indicated that these sequences had homology with the sequences present in bovine PAF-acetylhydrolase II (GenBank™/EBI Data Bank accession number D87559; 78.6%, 100%, 71.4%, 81.8%, and 100%, respectively).
The finding that the enzyme shared the sequence homology with bovine PAF-acetylhydrolase II led us to ask the question of whether the enzyme carried the activity of hydrolyzing PAF. We found that the PAF-dependent transacetylase also contains lysoPAF hydrolyzing activities in crude membrane fractions and was measured in the presence of 0.1% BSA-saline (3). These results suggest that the $K_m$ values are varied with different tissues and cell types. Nevertheless, these values are well below the physiological concentration of lysoplasmalagens in the cells. Calculation from the data obtained by Tessner et al. (16) and Nieto et al. (17) indicated that the lysoplasmalogen concentration could reach 0.5–0.7 mM when human neutrophils were stimulated with ionophore A23187 for 5 min.

In contrast, inhibition of sphingosine transacetylase activity (Fig. 4B) was resulted when sphingosine concentrations were higher than 50 $\mu$M. If the points during the ascending portion of the curve were used, the calculated $K_m$ for sphingosine was 4.1 $\mu$M. This value is similar to the $K_m$ reported for sphingosine of the purified rat kidney sphingosine kinase (5 $\mu$M) (18).

We have shown that, using mixed substrate experiments and crude membrane fractions from HL-60 cells (6), either CoA-independent transacetylase has a higher substrate affinity for sphingosine than any of the other substrate analogs, or the possibility of two isoforms of the transacetylase might be involved in the transfer of acetate from PAF to sphingosine and was measured in the presence of 0.1% BSA-saline (3).

The finding that the enzyme shared the sequence homology with bovine PAF-acetylhydrolase II led us to ask the question of whether the enzyme carried the activity of hydrolyzing PAF. We found that the PAF-dependent transacetylase also contains PAF hydrolyzing activities in crude membrane fractions and purified preparations; the ratios of lysoplasmalogen transacetylase/acyethylhydrolase were 0.74, 0.84, and 1.04 (average of two experiments with <10% variation) in the mitochondria, microsomes, and purified enzyme, respectively.

The reason for the slight increase in ratios of lysoplasmalogen transacetylase/acyethylhydrolase activities during purification is not clear at present. It is possible that some of the contaminating acetylhydrolase activities were removed during purification procedures or the presentation of substrates might differ between the purified enzyme and crude membrane-bound enzymes due to the possible interaction of membrane phospholipids with the lipid substrates.

**Kinetic Properties**—The dependence of enzyme activity on substrate concentration is shown in Fig. 4. PAF:lysoplasmalogen transacetylase activity displayed typical Michaelis-Menten kinetics, with an apparent $K_m$ of 227 $\mu$M for lysoplasmalogen and a $V_{max}$ of 81 $\mu$mol/min-mg (Fig. 4A). Interestingly, the $K_m$ for lysoplasmalogens was 106.4 $\mu$M in the membranes of HL-60 cells (3) and 80 $\mu$M in the homogenates of calf pulmonary artery endothelial cells (data not shown). These results suggest that the $K_m$ values are varied with different tissues and cell types. Nevertheless, these values are well below the physiological concentration of lysoplasmalagens in the cells. Calculation from the data obtaining by Tessner et al. (16) and Nieto et al. (17) indicated that the lysoplasmalogen concentration could reach 0.5–0.7 mM when human neutrophils were stimulated with ionophore A23187 for 5 min.

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**Effect of Inhibitors**—PAF:lysoplasmalogen transacetylase activity was inhibited by diisopropyl fluorophosphate in a dose-

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**Purification of PAF-dependent Transacetylase**

**FIG. 2.** SDS-PAGE of protein fractions isolated from different steps of purification of PAF-dependent transacetylase from rat kidney membranes. SDS-PAGE was carried out as described under “Experimental Procedures.” Amounts of transacetylase activities loaded onto each lane are listed in parentheses. Lane 1, mitochondrial fractions (1 nmol/min); lane 2, microsomal fractions (1 nmol/min); lane 3, solubilized fractions from mitochondria (1 nmol/min); lane 4, solubilized fractions from microsomal fractions (1 nmol/min); lane 5, DEAE-Sepharose (1 nmol/min); lane 6, hydroxyapatite (1 nmol/min); lane 7, phenyl-Sepharose (1 nmol/min); lane 8, chromotofocusing (2 nmol/min); lane 9, native-PAGE (4 nmol/min); lane 10, affinity gel (4 nmol/min). Molecular weight standards: bovine serum albumin ($M_r$ = 66,000), ovalbumin ($M_r$ = 45,000), glycer-aldehyde-3-phosphate dehydrogenase ($M_r$ = 36,000), carbonic anhydrase ($M_r$ = 29,000), trypsinogen ($M_r$ = 24,000), and trypsin inhibitor ($M_r$ = 20, 100).

**FIG. 3.** Determination of molecular weight of PAF-dependent transacetylase. The log molecular weight of standard proteins versus mobility on the SDS-PAGE was plotted. Molecular weight protein markers were the same as described in Fig. 2. The molecular mass of the purified transacetylase from rat kidney membranes is shown by the arrow.
Purification of PAF-dependent Transacetylase

Effects of inhibitors on PAF:lysoplasmalogen and PAF:sphingosine transacetylase activities purified from rat kidney membranes

Each inhibitor was incubated with the purified enzyme for 30 min at 37 °C, and then the enzyme reaction was started by adding the substrate.

| Inhibitors                  | mM       | Remaining enzyme activity (%)*a                |
|----------------------------|----------|-----------------------------------------------|
|                            |          | PAF:lysoplasmalogen transacetylase | PAF:sphingosine transacetylase |
| Disopropyl fluorophosphate | 0.1      | 33.3                                          | 45.9                           |
| Pefabloc                   | 1        | 10.4                                          | 15.6                           |
|                            | 0.1      | 7.0                                           | 3.4                            |
| DTNB‡                      | 0.1      | 38.3                                          | 6.9                            |
|                            | 1        | 56.0                                          | 13.6                           |
| NEM                        | 0.1      | 90.7                                          | 22.6                           |
|                            | 1        | 43.8                                          | 18.5                           |
| Diethyl pyrocarbonate      | 0.1      | 91.2                                          | 98.9                           |
|                            | 1        | 64.5                                          | 64.4                           |

a Values represented the average of duplicate determinations with variations <10%.

b Each substrate was dissolved in ethanol at the final concentration of 5% (v/v).

dependent manner (Table IV and data not shown). These data suggest that serine residue is involved in the active site of the enzyme. The specific inhibitor of plasma PAF-AH and serine esterase (19), p-aminooethyl benzenesulfonfyl fluoride (Pefabloc), completely inhibited PAF:lysoplasmalogen transacetylase at a concentration of 0.1 mM. These results are in good agreement with the fact that the transacetylase partially shared structural homology with PAF-AH II, and human PAF-AH II exhibited 43% predicted amino acid identity to human plasma PAF-AH (20). Both 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB) and N-ethylmaleimide (NEM), which react with the sulfhydryl group of cysteine, partially block the enzyme activity, indicating that sulfhydryl group is important for enzyme activity. Diethyl pyrocarbonate, a modifier of the histidine, at 1 mM partially inhibited the PAF:lysoplasmalogen transacetylase activity. In parallel, the PAF:sphingosine transacetylase activities were also affected by these inhibitors except with different sensitivities especially with the sulfhydryl reagents (Table IV).

DISCUSSION

PAF-dependent transacetylase was purified from rat kidney membrane 13,700-fold. The final preparation, which was judged to be nearly homogeneous by SDS-APGE, yielded a single protein band of 40 kDa. DTT was included throughout the purification because enzyme activity was rapidly decreased in the absence of DTT. DTT is thought to be necessary for protecting the sulphydryl group of cysteine from oxidation because DTNB and NEM partially abolish the enzyme activity (Table IV). NEM and DTNB inhibit PAF:lysoplasmalogen and PAF:sphingosine transacylases with different inhibitory potencies. For example, PAF:sphingosine transacylase activity (22.6% of enzyme activity remained after 30 min of 0.1 mM NEM treatment) was more susceptible to NEM than PAF:lysoplasmalogen transacylase (which retained 90.7% of enzyme activity after 0.1 mM NEM treatment for 30 min). These results are consistent with our previous work using HL-60 cell membranes as the enzyme source (6). This differential effect of NEM and DTNB on enzyme activities suggests that the sulphhydril group of cysteine play a more important role in the interaction of the enzyme with sphingosine, as compared with that of lysoplasmalogen.

In the present investigation, PAF-dependent transacetylase was isolated from mitochondrial and microsomal membrane fractions. However, PAF-dependent transacetylase activity is also present in cytosolic fraction (7). It is well known that some of the enzymes are distributed in both membrane and cytosols, and translocation of the enzymes is regulated by modification of the protein, such as phosphorylation (21) and myristoylation (22). For example, phosphorylation of cytosolic phospholipase A2 causes translocation from the cytosol to the membrane (21). We have demonstrated that PAF:acyllyso-GPC transacylase is regulated through phosphorylation/dephosphorylation (4). It is not known presently whether phosphorylation/dephosphorylation is involved in determining the cellular localization of PAF-dependent transacetylase or PAF-AH II is regulated through phosphorylation/dephosphorylation. The availability of a homogeneous preparation of transacylase protein will facilitate the development of specific antibodies against this enzyme. Specific antibodies against PAF-dependent transacylase will be useful in clarifying the relationships of the enzyme activities located in membrane and cytosol as well as the mechanism of translocation of this enzyme between these two fractions.

Analysis of the partial amino acid sequences of five tryptic-digested peptide fragments revealed that this enzyme shared sequence homology with the previously reported bovine cytosolic PAF-AH II (20). Cytosolic PAF-AH II was found to be N-myristoylated and could be translocated from cytosol to mem-
branes in oxidative stress-induced cells (23). In addition, purified PAF-dependent transacylase from rat kidney membranes contained PAF-AH activity in the absence of a lipid acceptor. The activities of both PAF-dependent transacylase and PAF-AH II (24) were inhibited by serine esterase inhibitors and sulfhydryl reagents. Preliminary results (unpublished data in collaboration with Dr. K. Inoue’s laboratory) also indicated that purified transacylase and transacylases present in the mitochondria and microsomes cross reacts with anti-human PAF-AH II monoclonal antibody. These results suggest that transacylase and PAF-AH II share similar structural requirement for the active site and the same recognition site for the immunological epitope. However, we have to wait for the result of cDNA cloning of the transacylase in order to compare the sequence of the transacylase with that of the PAF AH II. Information obtained from the amino acid sequences of the analyzed peptides of the PAF-dependent transacylase should provide the necessary means to identify the specific cDNA clones from the rat kidney cDNA library. It is possible that transacylase and PAF-AH II share the same amino acid sequences, but are differentially posttranslationally modified.

Regardless of structural similarity or difference between transacylase and PAF-AH II, our finding that transacylase has three distinct catalytic activities raise important questions concerning the regulation, biological implications, and consequences of this unique enzyme. It is possible that PAF may exert some of its biological effects through transacylase without the need or presence of participation of intracellular PAF receptor(s).

In addition to the PAF-dependent transacylase, there are other examples that an enzyme with transesterification activity also possesses hydrolytic activity. Lecithin-cholesterol acyltransferase (LCAT), which normally transfers the acyl group of PC to cholesterol, also hydrolyzes PAF (25). PAF-AH activity of LCAT plays the role of detoxification of oxidized PC, especially when the PAF-AH is absent or inactivated (25). Thus, PAF-degrading enzyme, LCAT plays the role of detoxification of oxidized PC, especially when the PAF-AH is absent or inactivated (25). Therefore, PAF-dependent transacylase is the enzyme responsible for the biosynthesis of C2-ceramide, and the cellular concentration of C2-ceramide is in the range (micromolar) that could exert significant biological effects. In addition, the signaling pathways utilized by the sphingomyelinase differs from those of cell-permeable ceramide analogs (31). Additionally, no detectable biological effects are observed in that neutral sphingomyelinase overexpressed cells (32). These results suggest that C2-ceramide should be classified as a naturally occurring novel lipid mediator. Furthermore, acyl analogs of PAF have biological characteristics distinct from that of PAF (4). Future research in this laboratory is directed toward the elucidation of the mechanism(s) of how a single enzyme controls and regulates three different kinds of enzymatic reactions. Additionally, progress is being made to generate cDNA and polyclonal antibodies against the transacylase.

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Purification of PAF-dependent Transacylase