Platelet-Activating Factor Acetylhydrolases: Broad Substrate Specificity and Lipoprotein Binding Does Not Modulate the Catalytic Properties of the Plasma Enzyme

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Received November 10, 2000

Abstract: Platelet-activating factor acetylhydrolases (PAF-AHs) are a group of enzymes that hydrolyze the sn-2 acetyl ester of PAF (phospholipase A2 activity) but not phospholipids with two long fatty acyl groups. Our previous studies showed that membrane-bound human plasma PAF-AH (pPAF-AH) accesses its substrate only from the aqueous phase, which raises the possibility that this enzyme can hydrolyze a variety of lipid esters that are partially soluble in the aqueous phase. Here we show that pPAF-AH has broad substrate specificity in that it hydrolyzes short-chain diacylglycerols, triacylglycerols, and acetylated alkanols, and displays phospholipase A1 activity. On the basis of all of the substrate specificity results, it appears that the minimal structural requirement for a good pPAF-AH substrate is the portion of a glyceride derivative that includes an sn-2 ester and a reasonably hydrophobic chain in the position occupied by the sn-1 chain. In vivo, pPAF-AH is bound to high and low density lipoproteins, and we show that the apparent maximal velocity for this enzyme is not influenced by lipoprotein binding and that the enzyme hydrolyzes tributyrlyglycerol as well as the recombinant pPAF-AH does. Broad substrate specificity is also observed for the structurally homologous PAF-AH which occurs intracellularly [PAF-AH(II)] as well as for the PAF-AH from the lower eukaryote Physarum polycephalum although pPAF-AH and PAF-AH(II) tolerate the removal of the sn-3 headgroup better than the PAF-AH from P. polycephalum does. In contrast, the intracellular PAF-AH found in mammalian brain [PAF-AH(Ib) α1/α1 and α2/α2 homodimers] is more selectively operative on compounds with a short acetyl chain although this enzyme also displays significant phospholipase A1 activity.

Platelet-activating factor (PAF; 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a phospholipid autacoid implicated in many allergic and inflammatory responses (reviewed in ref 1). PAF is produced upon diverse proinflammatory stimulation under tight enzymatic regulation and activates cells such as platelets, neutrophils, monocytes, macrophages, and smooth muscle cells through a PAF-specific G-protein coupled receptor. The biological actions of PAF via the PAF receptor can be mimicked by oxidized phospholipids produced by oxidative modification of membrane phospholipids and of low-density lipoproteins (LDL) via free radical reactions (2). Such oxidized phospholipids are involved in vascular inflammation and atherosclerosis (3, 4).

The bioactivities of PAF and oxidized phospholipids are abolished by the hydrolysis of their sn-2 ester by PAF-acetylhydrolases (PAF-AHs). PAF-AHs have been found in blood plasma and in the intracellular fluid of various cells and organisms (5–7). Plasma PAF-AH (pPAF-AH) is a 45-kDa protein associated primarily with small, dense LDL and high density lipoproteins (HDL) (8–10). It has been reported that 4% of the Japanese population lack pPAF-AH and that such a deficiency or decrease in pPAF-AH activity is a risk factor for severity in asthma and atopy (11–13). In animal models, recombinant pPAF-AH was effective in treating acute pancreatitis (14), asthma (15), anaphylactic shock (16), etc. Recombinant pPAF-AH is being tested in clinical trials with patients at risk for developing acute respiratory distress syndrome (17).

1 Abbreviations: C10-PAF, 1-decanoyl-2-acetyl-sn-glycero-3-phosphocholine; C14-PAF, 1-myristoyl-2-acetyl-sn-glycero-3-phosphocholine; C16-PAF, 1-0-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine; C18-PAF, 1-O-octadecyl-2-acetyl-sn-glycero-3-phosphocholine; 2-C16-PAF, 1-acetyl-2-O-hexadecyl-rac-glycero-3-phosphocholine; [3H]C10-PAF, 1-decanoyl-2-(1-[3H]acetyl)-sn-glycero-3-phosphocholine; [3H]-C16-PAF, 1-0-hexadecyl-2-1-[3H]acetyl)-sn-glycero-3-phosphocholine; CHAPS, (3-[3-cholamidopropyl]dimethylammonio]]-1-propanesulfonate); CMC, critical micelle concentration; EIA, enzyme immunoassay; HDL, high-density lipoproteins; LDL, low-density lipoproteins; PAF, platelet-activating factor; PAF-AH(II), human intracellular PAF acetylhydrolase isof orm II; pPAF-AH, human plasma PAF acetylhydrolase; Physarum PAF-AH, PAF acetylhydrolase from Physarum polycephalum; PC, phosphatidylcholine.
PAF-AH(II) is a 40-kDa, intracellular PAF-AH highly expressed in liver and kidney, and it shares 41% sequence identity with pPAF-AH. pPAF-AH and PAF-AH(II) do not distinguish between an ester or an ether at the sn-1 position of PAF or PAF analogues (18), and both can hydrolyze phospholipids with short to medium length sn-2 acyl chains including truncated chains derived from oxidative cleavage of long-chain polyunsaturated fatty acyl groups (18). Activity of these enzymes toward phospholipids with two long (14–18 carbons) fatty acyl chains is negligible (18–20).

Another intracellular form of PAF-AH, the PAF-AH( Ib) complex, has been found in brain. Bovine brain PAF-AH-( Ib) complex consists of two 26-kDa catalytic subunits, α1 and α2, which share 63% sequence identity with each other, and a regulatory 45-kDa β-subunit which is the product of the Miller–Dieker lissencephaly gene (21). The amino acid sequences of these subunits have poor homology with pPAF-AH or PAF-AH(II) (22). Biochemical analyses revealed that the α1/α2 heterodimer and the α2/α2 homodimer are the major catalytic units of embryonic and adult brain PAF-AHs, respectively (22). The α-subunits of PAF-AH( Ib) form catalytically active homodimers, α1/α1 and α2/α2, when individually expressed in E. coli. Previous studies have shown that PAF-AH( Ib) hardly hydrolyzes the PAF analogues with sn-2 propionyl and butyroyl moieties, displaying stricter specificity for the sn-2 acetyl group of a phosphoglyceride than does pPAF-AH or PAF-AH(II) (18).

A PAF-AH from the slime mold Physarum polycephalum (physarum PAF-AH) has been purified and cloned in an effort to understand PAF-hydrolyzing activities in lower organisms which may not possess PAF. This enzyme hydrolyzes oxidized phospholipids as well as PAF and has transacylase activity. It is a cytosolic enzyme that shows both cytosolic and membrane subcellular distribution; PAF-AH(II) behaves similarly (24). However, the amino acid sequence homology of physarum PAF-AH with other PAF-AHs is weak (24), and its substrate specificity has not been studied as extensively as the other PAF-AHs.

We have recently reported that vesicle-bound pPAF-AH necessarily accesses its substrates from the aqueous phase (25). In such a mechanism, the aqueous solubility of substrate is an important factor for controlling the substrate specificity of the enzyme. This plus the fact that pPAF-AH was first identified only on the basis of its ability to hydrolyze PAF but not phospholipids with long sn-2 chains suggested that this enzyme may hydrolyze a diverse set of water-soluble esters besides PAF. In the present study, we have explored this possibility and have also carried out a detailed comparison of the substrate specificities of pPAF-AH, PAF-AH(II), PAF-AH(Ib), and physarum PAF-AH. In addition, we have examined the effect of lipoprotein association on the specific activity and substrate specificity of pPAF-AH.

MATERIALS AND METHODS

Materials. All 1,2-diacyl-sn-phosphatidylcholines, 1,2-diacyl-sn-glycerols, dioctanoylthreeneuglycerol, and 1-oleoyl-2-acetyl-sn-glycerol are from Avanti Polar Lipids and are stereochemically pure unless noted otherwise. Methyl butyrate is from Aldrich. C16-PAF and C18-PAF are from BioMol. 3-O-Octadecyl-2-acetyl-sn-glycero-1-phosphocholine is from Bachem. [1-3H]Acetic anhydride and [3H]C16-PAF are from NEN Life Science Products. All lysophosphocholines, triacylglycerols, 1(3)-monoaoyl-rac-glycerols, butyl-, octyl-, acetates, gentamicin sulfate, diethylenetriaminepentaacetic acid, Antifoam A, and the Cholesterol Reagent are from Sigma. 2-Monoolein (2-oleoylmonoacylglycerol) is from Serdary Research Laboratories (London, Ontario). C10-PAF was prepared as described (25). Tributyrlyglycerol [1-butryl-1-14C] is from American Radiolabeled Chemicals Inc. Trolox is from Calbiochem. BCA (bicinchoninic acid) reagent, streptavidin-horseradish peroxidase, nitroblue tetrazolium chloride, and 5-bromo-4-chloro-3-indoly-phosphate-p-toluidine solution are from Promega. Sheep anti-human-apolipoprotein AI and B immunoglobulins are from Boehringer Mannheim Biochemica. Recombinant pPAF-AH was expressed in E. coli and purified as described (26). Recombinant forms of human PAF-AH(II), physarum PAF-AH, and bovine PAF-AH(Ib) α1/α1 and α2/α2 homodimers were purified as described (24, 27).

Synthesis of 1-Acetyl-2-O-hexadecyl-rac-phosphocholine (2-C16-PAF). 1-Hydroxy-2-O-hexadecyl-rac-phosphocholine was a generous gift from Dr. H. S. Hendrickson and E. K. Hendrickson (University of Washington). Acetylation of this lysophospholipid (0.1 mmol) was done with acetic anhydride (~0.5 mmol) in 5 mL of CH2Cl2 containing 4-(N,N-dimethy lamino)pyridine (0.1 mmol) (28), and the product was purified by flash chromatography over 2 g of silica gel with a chloroform/methanol/water step gradient as follows: 150 mL of chloroform/methanol (90:10 v/v), 100 mL of chloroform/methanol (80:20), 100 mL of chloroform/methanol (65:35), 20 mL of chloroform/methanol/water (65:35:1), 20 mL of chloroform/methanol/water (65:35:2), 20 mL of chloroform/methanol/water (65:35:3), 40 mL of chloroform/methanol/water (65:35:4), and 36 mL of chloroform/methanol/ammonia/water (20:14:1:1). Fractions containing PC were pooled, and solvent was removed with a rotary evaporator. The purity and the structure of the product were confirmed by TLC and NMR. The amount of phosphate was determined using the Ames method (29).

Synthesis of [3H]Cl10-PAF. Acetylation of 1-decanoyl-2-hydroxy-sn-glycero-3-phosphocholine (0.10 mmol) was done with [1-3H]acetic anhydride (0.25 mmol, 50.00 mCi/mmol, NEN Life Science Products) in 10 mL of CH2Cl2 containing 4-(N,N-dimethylamin)pyridine (0.48 mmol) at room temperature for 6 h. The product was purified over silica gel as described for the nonradioactive synthesis of C10-PAF (25, 28). The purity was confirmed by TLC followed by fluo rography using EN3HANCER spray (NEN), and the amount of the product was determined by liquid scintillation counting.

Titrimetric PAF-AH Assay. The standard titrimetric pH-stat assay was done as previously described (30) at 22 ± 1 °C unless noted otherwise. The reaction mixture contained substrate at the desired concentration in 5 mL of 1 mM NaCl solution, and the reaction was initiated by adding enzymes diluted from stock solutions. While the amount of enzymes used varied (0.24–20 μg) depending on the substrate, a fixed amount was employed for one substrate as the substrate concentration was varied. The stock solution of recombinant pPAF-AH was prepared in 50 mM sodium phosphate, pH 7.5, 125 mM NaCl, 0.1% Pluronic F68, and 0.02% Tween 80 at an enzyme concentration of 4 mg/mL. Stock solutions of other PAF-AHs were prepared in 10 mM Tris-HCl, pH
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min as determined by Western blot analysis (see below). apolipoprotein AI-containing lipoproteins (HDL) and albumin solution (Pharmacia). Total cholesterol concentration was measured using the enzymatic endpoint reagent kit (Sigma).

Isolation of Plasma Lipoprotein Fractions by KBr-Density Gradient Ultracentrifugation. HDL and LDL were prepared by a single, discontinuous density gradient ultracentrifugation using a KBr gradient (34). The KBr density gradient was used instead of the D2O density gradient to separate a broader range of lipoproteins. Plasma (200 μL) was mixed with 1.8 mL of KBr–PBS solution (d = 1.38 g/mL), and 1.9 mL of the mixture was layered underneath 3.5 mL of PBS solution (d = 1.006 g/mL) in a Beckman Ultraclear centrifuge tube (0.5 × 2 in). The ultracentrifugation was carried out in a Beckman SW55Ti rotor at 55 000 rpm (368000gav) at 10 °C for 3 h. Fractions of 0.23 mL were collected by eluting the solution from the bottom using a peristaltic pump at 1 mL/min.

To check the effect of KBr on pPAF-AH activity and EIA, pooled fractions of HDL (fractions 7–9; d = 1.14–1.18 g/mL) and LDL (fractions 12–14; d = 1.03–1.06 g/mL) were dialyzed overnight at 4 °C against phosphate-buffered saline (8.1 mM Na2HPO4, 1.5 mM KH2PO4, 137 mM NaCl, 2.7 mM KCl, 1 mM EDTA, and 0.1 mM diethylenetriaminepentaacetic acid, pH 7.5), and the radiometric pPAF-AH assay and EIA were done on the dialyzed fractions and on the undialyzed ones. KBr did not alter the results of the assays, and the recovery after dialysis was ~90% for both the pooled HDL and LDL fractions. Thus, undialyzed fractions were used for further studies.

Radiometric PAF-AH Assay. Radiometric assays for pPAF-AH were performed as previously described (35) with minor modification using a Biomek 2000 automatic pipetting station with 96-well microplates. The assay buffer (pPAF-AH assay buffer) was composed of 25 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and 1 mM EDTA with a specified concentration of CHAPS and [3H]C16-PAF, [3H]C10-PAF, or tributyroylglycerol[butyric-1-14C] in a total volume of 100 μL. The reaction was initiated by the addition of 20 μL of enzyme solution previously diluted in the same buffer as the substrate. At least three different dilutions of enzyme solution were included in one plate. The incubation was done at 37 °C for 15 min, and the reaction was quenched by adding 125 μL of activated charcoal suspension (25 mg/mL). The plate was centrifuged at 2700 rpm (1400gav) for 10 min at 5 °C in a Beckman GS-6r centrifuge using a Microplate carrier for a Beckman GH 3.7 rotor. Radioactivity in the supernatant was counted in a Wallac Microbeta liquid scintillation counter to determine the amount of free [1-3H]acetic acid. Only the data giving less than 30% of total substrate hydrolysis were used to determine the initial rates, and the results are expressed as micromoles of substrate hydrolyzed per hour per milliliter of enzyme solution after correction for nonenzymatic degradation of the substrate.

When the activity of exogenously added recombinant pPAF-AH was measured, 10 μL of a stock solution of purified recombinant pPAF-AH (8.1 nM in 25 mM Tris-
HCl, pH 7.5, 0.5 M NaCl, 1 mM EDTA, and 6 mM CHAPS) was added to 100 μL of each lipoprotein fraction sample, and the mixture was incubated for 30 min at room temperature and then processed as described above.

Quantitative Enzyme Immunoassay (EIA) of pPAF-AH. A 96-well polystyrene plate (Immulon 4 “C” Bottom Divida-strip plate) was coated with purified anti-recombinant pPAF-AH murine monoclonal antibody, 90G11D (prepared by the in-house facility at ICOS, 125 μL/well, 2.5 μg/mL in 50 mM sodium carbonate/bicarbonate pH 9.6, 0.05% Proclin 300 (Supelco) at 37 °C for 2 h. The coating solution was removed, and the plate was incubated with blocking solution (300 μL/well, 50 mM sodium citrate, pH 7.4, 145 mM NaCl, 25 g/L dry nonfat milk, 0.005% Antifoam A, and 0.05% Proclin 300) at room temperature for 30 min. Samples were diluted 6- to 101-fold, and standards were prepared at eight different concentrations between 0 and 10 ng/mL (0–0.22 nM) from a recombinant pPAF-AH stock solution (4 mg/mL in 50 mM sodium phosphate, pH 7.5, 125 mM NaCl, 0.1% Pluronic F68, and 0.02% Tween 80). The same diluent buffer was used for both samples and standards (50 mM sodium citrate, pH 7.4, 145 mM NaCl, 7.5 mM CHAPS, 50 g/L dry nonfat milk, 2.5% horse serum, 0.005% Antifoam A, 0.05% Tween 20, 0.05% Proclin 300, and 0.015% Blue food color). Sample or standard solution (100 μL/well) was added to the plate after the removal of the blocking solution, and the plate was incubated at 37 °C for 30 min. The plate was then washed 5 times with 145 mM NaCl, 0.05% Tween 20 using a microplate washer (Bioteck EL403H). The secondary antibody, biotinylated anti-recombinant pPAF-AH murine monoclonal antibody, 90F2D (prepared by the in-house facility at ICOS) was added (100 μL/well in the biotin conjugate buffer made of 20 mM sodium citrate, pH 7.0, 0.5 M NaCl, 6.3 g/L dry nonfat milk, 0.25 g/L goat gamma globulin, 0.005% Antifoam A, 0.2% Tween 20, 0.05% Proclin 300, and 0.025% Red food color), and the plate was incubated at 37 °C for 30 min. The plate was washed as previously described and incubated with streptavidin-hors eradish peroxidase (Promega, 100 μL/well, 0.5 μg/mL in the biotin conjugate buffer) at 37 °C for 30 min, followed by another wash step. Subsequently, substrate solution for the peroxidase (100 μL/well, 0.1 M sodium acetate, pH 5.5, 0.015% hydrogen peroxide, and 0.42 mM tetramethylbenzidine) was added, and the plate was incubated at room temperature for 30 min in the dark. The reaction was stopped by adding 100 μL of 1 N H₂SO₄. The absorbance of each well was read at 450 and 630 nm using a microplate reader (SpectraMax 250). The difference A₄₅₀–A₆₃₀ was used for calculation. Only the sample data that gave values within the standard range were used.

RESULTS

Substrate Specificity of pPAF-AH. The substrate specificity of pPAF-AH was examined by measuring the initial hydrolysis rates of various substrates at different concentrations using a pH-stat as described in Materials and Methods. Hyperbolic kinetics (apparent saturation) was observed in the velocity versus substrate concentration plots (not shown), and the apparent Vₘₐₓ (Vₘₐₓ,app) and Kₘₐₓ (Kₘₐₓ,app) values were obtained from the fit of the data to the classical Michaelis–Menten equation. In some cases, the Kₘₐₓ,app value was too low to measure with the pH-stat assay (Table 1). As described previously (25), hyperbolic kinetics does not necessarily imply saturation of the active site of pPAF-AH with substrate. To a first approximation, the concentration of substrate in the aqueous phase cannot exceed its CMC. Thus, in the context of the aqueous phase mechanism for pPAF-AH, the enzymatic rate cannot increase further once the concentration of substrate in the aqueous phase reaches a constant amount determined by its solubility. This is true in the presence and absence of a vesicle or lipoprotein interface to which pPAF-AH may be bound. Thus, it is important to refer to the kinetic parameters derived from the velocity versus substrate concentration plots as apparent kinetic values. In addition, the binding of pPAF-AH to vesicles of phosphatidylcholine or phosphatidyl ethanol does not significantly alter the catalytic efficiency of the enzyme toward PAF and PAF analogues in the aqueous phase, i.e., little interfacial activation (25). Thus, we decided to carry out substrate specificity studies of this enzyme in the absence of a phospholipid interface.

As shown in Table 1, Vₘₐₓ,app for the hydrolysis of C16-PAF by pPAF-AH is 4-fold higher than that for C18-PAF hydrolysis, presumably due to the higher aqueous phase solubility of the former as compared to the latter. pPAF-AH displays poor activity toward phospholipids with 16- to 18-carbon long sn-1 chains and an sn-2 chain of 8 carbons (without oxygen atoms from oxidation) (19, 25). We reasoned, in terms of the aqueous phase mechanism for pPAF-AH action, that hyperbolic kinetics does not necessarily imply saturation of the active site of pPAF-AH with substrate. To a first approximation, the concentration of substrate in the aqueous phase cannot exceed its CMC. Thus, in the context of the aqueous phase mechanism for pPAF-AH, the enzymatic rate cannot increase further once the concentration of substrate in the aqueous phase reaches a constant amount determined by its solubility. This is true in the presence and absence of a vesicle or lipoprotein interface to which pPAF-AH may be bound. Thus, it is important to refer to the kinetic parameters derived from the velocity versus substrate concentration plots as apparent kinetic values. In addition, the binding of pPAF-AH to vesicles of phosphatidylcholine or phosphatidyl ethanol does not significantly alter the catalytic efficiency of the enzyme toward PAF and PAF analogues in the aqueous phase, i.e., little interfacial activation (25). Thus, we decided to carry out substrate specificity studies of this enzyme in the absence of a phospholipid interface.

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<table>
<thead>
<tr>
<th>Substrate</th>
<th>Vₘₐₓ,app (s⁻¹)</th>
<th>Kₘₐₓ,app (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18-PAF</td>
<td>6.6 ± 0.5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>C16-PAF</td>
<td>26 ± 1</td>
<td>&lt;5</td>
</tr>
<tr>
<td>dibutyrlyl-PC</td>
<td>12 ± 1</td>
<td>4700 ± 700</td>
</tr>
<tr>
<td>diocanoyl-PC</td>
<td>25 ± 1</td>
<td>47 ± 9</td>
</tr>
<tr>
<td>didecanoyl-PC</td>
<td>5.3 ± 0.2</td>
<td>&lt;5</td>
</tr>
<tr>
<td>1-octadecyl-2-acetate-1-phosphocholine</td>
<td>&lt;0.2</td>
<td>not determined</td>
</tr>
<tr>
<td>3-octadecyl-2-acetate-1-phosphocholine</td>
<td>&lt;0.2</td>
<td>not determined</td>
</tr>
<tr>
<td>octadecylacetate</td>
<td>&lt;0.2</td>
<td>not determined</td>
</tr>
<tr>
<td>butyl acetate</td>
<td>0.96 ± 0.12</td>
<td>63000 ± 2200</td>
</tr>
<tr>
<td>octyl acetate</td>
<td>0.41 ± 0.02</td>
<td>55 ± 10</td>
</tr>
<tr>
<td>2-C16-PAF</td>
<td>8.1 ± 0.6</td>
<td>&lt;5</td>
</tr>
<tr>
<td>3-octadecyl-2-acetate-1-phosphocholine</td>
<td>&lt;0.2</td>
<td>not determined</td>
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</table>
with the diacyl-PC series of increasing fatty acyl chain length show that the enzyme prefers substrates that are more hydrophobic than dibutyroyl-PC, within the limit set by aqueous phase solubility of the substrate.

In our previous study (25), we found that pPAF-AH does not significantly discriminate against PAF analogues in which the choline portion of the polar headgroup is replaced with ethanolamine, glycerol, serine, and hydrogen. The relatively small differences in the rate of hydrolysis of this series of substrates are controlled mainly by their aqueous phase solubility (25). In the present study, we explored the requirement of the sn-3 phosphate group of PAF for recognition by pPAF-AH. As shown in Table 1, the diacylglycerols, 1-oleoyl-2-acetyl-sn-glycerol and 1,2-dioctanoyl-sn-glycerol, are all efficiently hydrolyzed by pPAF-AH.

Values of \( V_{\text{max,app}} \) for the diacylglycerols were 5–10-fold lower than the values for the corresponding phosphocholines, presumably due to the lower aqueous phase solubility of diacylglycerols versus the diacylphosphocholines. For example, the CMC of dihexanoyl glycerol (0.54 mM) is 14-fold lower than the CMC of dihexanoyl-PC (7.6 mM) (37). Dioctanoylethylene glycerol lacking the sn-3 -CH\(_2\)-OH of 1,2-dioctanoyl-sn-glycerol showed a ~12-fold lower rate than 1,2-dioctanoyl-sn-glycerol, which may also be due to the lower aqueous phase solubility of dioctanoylethylene glycerol resulting from the absence of the polar CH\(_2\)-OH moiety. These results show that a phosphate headgroup is not an essential part of PAF for recognition by pPAF-AH, while an sn-3 polar headgroup improves the efficiency of substrate hydrolysis presumably by increasing the solubility of the substrate in the aqueous phase.

Despite the higher aqueous phase solubility of monoacylglycerols versus diacylglycerols [for example, the CMC of monohexanoyl glycerol is \( \approx 200 \) times higher than that of dihexanoyl glycerol (37)], the racemic mixtures of 1- and 3-monoacylglycerols displayed 9–16-fold lower values of \( V_{\text{max,app}} \) than the corresponding diacylglycerols (Table 1). These results again support the notion that pPAF-AH prefers more hydrophobic substrates, within the limit of aqueous phase solubility. The hydrolysis of 2-monoolein was not detectable (not shown).

In support of the above observations showing that a polar headgroup is not required for pPAF-AH substrates, we found that triacylglycerols with short acyl chains, triacylglycerol, and tributyroylglycerol are efficiently hydrolyzed by pPAF-AH (Table 1). Trioctanoylglycerol is poorly hydrolyzed presumably because of its low aqueous phase solubility. The relatively high \( K_{\text{m,app}} \) for triacylglycerol as compared to that of tributyroylglycerol again supports the preference of pPAF-AH for more hydrophobic substrates. No attempt was made to determine the immediate product of trioctanoylglycerol hydrolysis since the initially formed dioctanoylglycerol is expected to be a good substrate for pPAF-AH. This was apparent from the observation that a lag phase was seen during trioctanoylglycerol hydrolysis in the first few minutes of the reaction (not shown). The simple esters such as methyl butyrate, butyl acetate, and octyl acetate are all substrates for pPAF-AH, although values of \( K_{\text{m,app}} \) for the less hydrophobic esters, methyl butyrate, and butyl acetate, are in the millimolar range (Table 1).

We also found that the isomer of PAF in which the acetyl group is at the sn-1 position and the hexadecyl ether is at the sn-2 position, 2-C16-PAF, is a good substrate for pPAF-AH, comparable to C16-PAF (Table 1). This clearly shows that pPAF-AH has phospholipase A\(_1\) activity. Although 2-C16-PAF is racemic, pPAF-AH is able to hydrolyze all of the phospholipid present in the reaction mixture with no anomalous change in rate beyond 50% hydrolysis. Thus, both enantiomers are efficiently hydrolyzed. We also studied the enantiomer of C18-PAF, 3-octadecyl-2-acetyl-sn-glycerol-1-phosphocholine, and found that it was hydrolyzed very poorly, if at all, by pPAF-AH (<0.2 s\(^{-1}\)) when tested at a concentration of 100 \( \mu \text{M} \).

**Substrate Specificities of PAF-AH(II), PAF-AH(Ib), and Physarum PAF-AH.** The substrate specificities of other PAF-AHs were examined by comparing the hydrolysis rates for several compounds tested at concentrations sufficient to give \( V_{\text{max,app}} \) with pPAF-AH. Figure 1, panel A, shows the comparison of the action of five PAF-AHs acting on PAF and PAF analogues containing an ether versus an ester linked sn-1 chain or with an extended sn-2 fatty acyl chain. The turnover numbers for pPAF-AH, PAF-AH(II), PAF-AH(Ib) \( \alpha_2/\alpha_1, \) PAF-AH(Ib) \( \alpha_1/\alpha_1 \) and physarum PAF-AH acting on 50 \( \mu \text{M} \) C14-PAF are 43, 40, 4.8, 0.6, and 103, respec-

**FIGURE 1:** Substrate specificity comparison of various PAF-AHs. (A) Relative rates for the hydrolysis of PAF analogues by PAF-AHs. The concentration of all substrates used was 100 \( \mu \text{M} \). (B) Comparison of pPAF-AH and PAF-AH(II). The concentrations of substrates used were 50 \( \mu \text{M} \) for C14-PAF, 2-C16-PAF, and 1,2-dioctanoyl-sn-glycerol, 79 \( \mu \text{M} \) for dioctanoyl-PC, and 160 \( \mu \text{M} \) for tributyroylglycerol. (C) Comparison of pPAF-AH and physarum PAF-AH. The concentrations of substrates used were 50 \( \mu \text{M} \) for C14-PAF, 2-C16-PAF, and 1,2-dioctanoyl-sn-glycerol, 100 \( \mu \text{M} \) for C16-PAF, 1-palmitoyl-2-acetyl-PC, 1-palmitoyl-2-propionyl-PC, and C18-PAF, 157 \( \mu \text{M} \) for dioctanoyl-PC, 1980 \( \mu \text{M} \) for 1(3)-monooctanoyl-1-acyl-glycerol, and 319 \( \mu \text{M} \) for tributyroylglycerol.
tively. For all enzymes, the rates for hydrolysis of C16-PAF and 1-palmitoyl-2-acetyl-PC are not significantly different (<1.6-fold), showing little discrimination between an ester and an ether at the sn-1 position of PAF. Increasing the length of the sn-2 chain from acetyl to propionyl gave different effects depending on the enzyme. As compared to C16-PAF, physarum PAF-AH hydrolyzed 1-palmitoyl-2-propionyl-PC at a similar rate, pPAF-AH and PAF-AH(II) were about 2-fold less active on 1-palmitoyl-2-propionyl-PC, and PAF-AH(Ib) α2/α2 was about 4-fold less active on 1-palmitoyl-2-propionyl-PC as compared to C16-PAF (Figure 1, panel A). All of these four PAF-AHs show a decrease in activity as the sn-1 chain length was increased (compare C16-PAF to C18-PAF in Figure 1, panel A). The turnover numbers for PAF-AH(Ib) α1/α1 acting on 1-palmitoyl-2-propionyl-PC and C18-PAF (< 0.1 s⁻¹) were significantly lower than those for the α2/α2 homodimer acting on the same substrates and are not determined.

As shown in Figure 1, panel B, the substrate specificity profile for pPAF-AH and PAF-AH(II) toward lipid esters with and without a phosphate are very similar. The results show that PAF-AH(II), like pPAF-AH, has significant phospholipase A₁ activity as well as significant activity toward short-chain diacyl- and triacyl-glycerols.

The specific activities of PAF-AH(Ib) α2/α2 acting on 2-C16-PAF and C16-PAF are comparable, 1.1 ± 0.1 and 3.2 ± 0.4 s⁻¹, respectively, showing that the brain enzyme also has significant phospholipase A₁ activity (Table 2). The specific activity of PAF-AH(Ib) α2/α2 for 1-octanoyl-2-acetyl-sn-glycerol is 13-fold lower than that for C16-PAF, which is similar to 8-fold difference shown by pPAF-AH for the same substrates (Table 2). However, in contrast to pPAF-AH and PAF-AH(II), PAF-AH(II) α2/α2 has relatively low activity toward tributyrinylglycerol, 1(3)-monooctanoyl-rac-glycerol, dioctanoylglycerol, and diacyl-PC (turnover numbers < 0.1 s⁻¹, more than 32-fold lower than the turnover number for C16-PAF). In addition, PAF-AH(Ib) α2/α2 hydrolyzes octyl acetate at a rate comparable to (1.7-fold lower) that for C16-PAF, and triacylglycerol at a rate higher (12-fold) than that for C16-PAF. In contrast, pPAF-AH shows 88- and 1.5-fold lower rates for octyl acetate and triacylglycerol, respectively, than that for C16-PAF (Table 2). PAF-AH(Ib) α1/α1 hydrolyzes octyl acetate and triacylglycerol 2- and 300-fold better than it does C16-PAF (Table 2), while it showed barely detectable hydrolysis (<0.1 s⁻¹) of tributyrinylglycerol, 1(3)-monooctanoyl-rac-glycerol, dioctanoylglycerol, dioctanoyl-PC, C18-PAF, 2-C16-PAF, and 1-octanoyl-2-acetyl-sn-glycerol. All together, the results show that PAF-AH(Ib) α2/α2 and α1/α1 have a strong preference for a short acetyl chain at a hydrolyzable position.

As shown in Figure 1, panel C, physarum PAF-AH, like pPAF-AH and PAF-AH(II), is able to hydrolyze a variety of lipid esters including dioctanoyl-PC and 2-C16-PAF (phospholipase A₁ activity). As for pPAF-AH and PAF-AH(II), 1(3)-monooctanoyl-rac-glycerol is a poor substrate. Physarum PAF-AH has greater preference for substrates with an sn-3 PC headgroup than PAF-AH and PAF-AH(II), as 1,2-dioctanoyl-sn-glycerol and tributyrinylglycerol show relatively very low hydrolysis rates compared to substrates containing PC.

**Studies with Lipoprotein-Bound pPAF-AH.** Since pPAF-AH is bound to lipoproteins in plasma, it is of interest to determine if lipoprotein binding modifies the kinetic behavior of this enzyme. When the distribution of pPAF-AH among lipoproteins was examined after isolating the HDL and LDL fractions by KBr-density gradient ultracentrifugation as described in the Materials and Methods, most of the PAF-AH immunoreactivity (70%) was found with LDL (d = 1.04–1.07 g/mL) and the rest with HDL (d = 1.14–1.18 g/mL) (Figure 2) as previously reported (8). The distribution of pPAF-AH among LDL density subfractions was also investigated after fractionation by the D₂O-density ultracentrifugation procedure as described in Materials and Methods. The highest PAF-AH activity was detected in the dense LDL fractions (d = 1.043–1.046 g/mL) (Figure 3), which is similar to the previous findings (9).

We also examined the distribution of endogenous pPAF-AH in plasma in the presence of varying concentrations of CHAPS to determine the detergent concentration required to dissociate the pPAF-AH from the lipoproteins. When plasma was chromatographed on a Superdex 200 size exclusion column in buffers containing varying concentra-

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**Table 2: Substrate Specificity of pPAF-AH and PAF-AH(lb)**

<table>
<thead>
<tr>
<th></th>
<th>pPAF-AH</th>
<th>PAF-AH(lb) α2/α2</th>
<th>PAF-AH(lb) α1/α1</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16-PAF 50 μM</td>
<td>24.6 ± 3.9</td>
<td>3.2 ± 0.4</td>
<td>0.34 ± 0.09</td>
</tr>
<tr>
<td>2-C16-PAF 50 μM</td>
<td>8.1 ± 0.6</td>
<td>1.1 ± 0.1</td>
<td>ND</td>
</tr>
<tr>
<td>C18-PAF 50 μM</td>
<td>4.8 ± 0.7</td>
<td>0.68 ± 0.12</td>
<td>ND</td>
</tr>
<tr>
<td>1-octanoyl-2-acetyl-sn-glycerol 50 μM</td>
<td>3.0 ± 0.4</td>
<td>0.25 ± 0.07</td>
<td>ND</td>
</tr>
<tr>
<td>octyl acetate 495 μM</td>
<td>0.28 ± 0.06</td>
<td>1.9 ± 0.3</td>
<td>0.69 ± 0.14</td>
</tr>
<tr>
<td>triacylglycerol 100 mM</td>
<td>16.5 ± 3.9</td>
<td>37.0 ± 4.7</td>
<td>101.3 ± 14.8</td>
</tr>
</tbody>
</table>

*The numbers are the apparent turnover numbers expressed in units of s⁻¹.*
assay with 100 activity in each fraction was determined by the radiometric recombinant pPAF-AH antibody, and the pPAF-AH enzymatic AH protein in each fraction was measured by EIA using anti-AH in the presence of 6 mM CHAPS. The amount of pPAF-AH was found to have similar specific activity (value of 290 ± 8 s⁻¹ for [3H]C16-PAF was obtained. This value is virtually identical to that of endogenous lipoprotein-bound pPAF-AH or to that for endogenous plus exogenously added recombinant pPAF-AH (Figure 5, panel B). All together, the results (Figures 2 and 5) show that endogenous pPAF-AH is not activated by binding to LDL or HDL. Further, endogenous pPAF-AH is not activated by binding to another protein component that may remain bound to LDL by CHAPS. Similar experiments as in Figure 5, panels A and B, were also carried out using 1.1 mM [3H]C10-PAF instead of [3H]C16-PAF to monitor pPAF-AH activity (Figure 5, panel C). Studies at lower substrate concentrations establish that Vmax,app was being measured for all pPAF-AH samples in the presence of 1.1 mM [3H]C10-PAF. Again, LDL-bound pPAF-AH was found to have similar specific activity (average value across LDL fractions of 314 ± 83 s⁻¹) as the specific activity after an amount of recombinant enzyme was exogenously added (337 ± 21 s⁻¹) and as recombinant

tions of CHAPS, lipoproteins eluted in the same fractions (LDL in fractions 15–20, HDL in fractions 23–28) in the presence of 0–6 mM of CHAPS as shown by the cholesterol and protein distributions (Figure 4, panels A and B). On the other hand, most of the pPAF-AH activity that eluted with LDL (85–90%) and HDL (10–15%) in the presence of 0–2 mM CHAPS (Figure 4, panel A) appeared in the fractions containing nonlipoprotein plasma proteins (fractions 28–32) in the presence of 6 mM CHAPS (Figure 4, panel B). These results show that 6 mM CHAPS was sufficient to fully dissociate pPAF-AH from the lipoproteins without noticeable disaggregation of the lipoproteins.

To compare the activity of lipoprotein-bound and CHAPS-solubilized pPAF-AH, we determined the specific activity of endogenous pPAF-AH in HDL fractions and in LDL subfractions in the presence and absence of 6 mM CHAPS. We also measured the specific activity of recombinant pPAF-AH in the presence of 6 mM CHAPS. The amount of pPAF-AH protein in each fraction was measured by EIA using anti-recombinant pPAF-AH antibody, and the pPAF-AH enzymatic activity in each fraction was determined by the radiometric assay with 100 µM [3H]C16-PAF in the presence and absence of 6 mM CHAPS. These numbers were used to obtain the specific activity, expressed in units of s⁻¹. The specific activity of pPAF-AH under all these conditions did not change when the [3H]C16-PAF concentration was decreased to 25 µM. Thus, with 100 µM [3H]C16-PAF, Vmax,app is being measured. As shown in Figure 2, the specific activity of endogenous lipoprotein-bound pPAF-AH measured with [3H]-C16-PAF in the absence of CHAPS is virtually constant across the HDL and LDL fractions. The specific activity of endogenous pPAF-AH does not change after dissociating the enzyme from LDL by the addition of 6 mM CHAPS (Figure 5, panels A and B). Furthermore, the specific activity after adding an amount of recombinant pPAF-AH comparable to the endogenous amount in the dense LDL fraction (d = 1.043–1.046 g/mL) is virtually identical to that measured for endogenous pPAF-AH in each LDL fraction (Figure 5, panels A and B). These results show that endogenous pPAF-AH is not activated by binding to LDL.

The specific activity of recombinant pPAF-AH acting on

**Figure 3:** Distribution of LDL and pPAF-AH from isopycnic density gradient ultracentrifugation using a D₂O gradient. Plasma was ultracentrifuged in a D₂O–H₂O density gradient as described in Materials and Methods. Fractions of 0.40 mL were collected and each was assayed for protein (Δ), cholesterol (■), and pPAF-AH protein determined by EIA (bar graph).

**Figure 4:** Dissociation of pPAF-AH from lipoproteins in the presence of CHAPS. Gel filtration chromatography was performed on 200 µL of plasma (~10 mg of total protein) in pPAF-AH assay buffer (25 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 1 mM EDTA) containing 0 mM or 2 mM CHAPS (A) or 6 mM CHAPS (B) over a Superdex 200 HR 10/30 column (10 mm × 300 mm, Pharmacia) at a flow rate of 0.5 mL/min. The volume of each fraction was 0.5 mL. Fractions were assayed for protein (Δ), cholesterol (■), and pPAF-AH activity (● for 0 and 6 mM, X for 2 mM) as described in Materials and Methods. Arrows indicate the elution positions of the molecular weight standards: thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), vitamin B-12 (1.3 kDa).
As noted above, recombinant pPAF-AH is able to efficiently hydrolyze tributyroylglycerol. To determine if LDL-bound pPAF-AH also displays high activity toward short chain triacylglycerols, we developed a radiometric assay using tributyroylglycerol[butyric-1-14C]. Data in Figure 5, panel D, show that LDL-bound endogenous pPAF-AH (Δ) and of total pPAF-AH after adding 3.7 ng (final concentration, 0.74 nM) of recombinant pPAF-AH (●) in each lipoprotein fraction in the absence of CHAPS was calculated from the data in panel A. The dotted line shows the specific activity of recombinant pPAF-AH in 6 mM CHAPS in the absence of lipoproteins (●) and with 3.7 ng (final concentration, 0.74 nM) of exogenously added recombinant pPAF-AH (●). The amount of endogenous pPAF-AH protein (EIA assay) is also shown (○). (D) The same LDL fractions as in panel A, were assayed for the hydrolysis of 17 μM (4.9 mCi/mmol), 168 μM (4.9 mCi/mmol), 293 μM (2.8 mCi/mmol) tributyroylglycerol[butyric-1-14C] in the absence of CHAPS. The activity of 0.90 nM recombinant pPAF-AH is shown on the right as a reference.

**DISCUSSION**

**Substrate Specificities of Multiple PAF-AHs.** Our previous study (25) showing that vesicle-bound pPAF-AH necessarily accesses its substrate from the aqueous phase raises the possibility that this enzyme can hydrolyze a number of esters other than PAF and closely related analogues that are considerably more soluble in the aqueous phase than are phospholipids with long sn-1 and sn-2 fatty acyl chains. As a prelude, we showed that the enzyme does not significantly recognize the choline group attached to the sn-3 phosphosphate of PAF (25). In the present study, we show that even the sn-3 phosphate is not required; 1-oleoyl-2-acetyl-sn-glycerol is a good pPAF-AH substrate. On the basis of all of the substrate specificity results, it appears that the minimal structural requirement for a good pPAF-AH substrate is the portion of a glyceride derivative that includes an sn-2 ester and a reasonably hydrophobic chain in the position occupied by the sn-1 chain (Figure 6). No group in the sn-3 position is required. In the context of this model, dibutyroyl-PC binds...
AH as a phospholipase A\textsubscript{2} (\(\alpha/\beta\) hydrolase reminiscent of a neutral lipase, distinct from the other known phospholipase A\textsubscript{2}s which do not conform to the \(\alpha/\beta\) hydrolase model (26, 39). Some researchers have used the name, lipoprotein-associated lipase for the enzyme (40, 41), but it is important to note that lipases are interfaceal enzymes whereas pPAF-AH is not.

The substrate specificity of pPAF-AH and PAF-AH(II) are very similar, suggesting that their physiological functions may be partially overlapping. The broad specificity of these enzymes raises the possibility that PAF and phospholipids with oxidatively truncated sn-2 chains are not the only physiological substrates for these enzymes. Although the concentrations of endogenous PAF in the blood stream of normal and pPAF-AH-deficient individuals have not been measured, a previous study showed that exogenously added PAF (10\(^{-9}\) M) has a half-life of 5 min in the plasma from normal subjects, whereas more than 95% of the PAF remained after 15 min in the plasma from pPAF-AH-deficient subjects (42). This result provides evidence that PAF is one of the in vivo substrates for pPAF-AH. Previous studies on PAF-AH(II) have shown that the enzyme senses the redox state of the cell and protects cells against oxidative stress-induced cell death presumably through the hydrolysis of oxidized phospholipids (43). Although it is questionable that the compounds used in this study are present in vivo, the structural requirements of a good substrate for the enzymes seem to be compatible with the anti-oxidative scavenger role of both enzymes. It seems reasonable for an enzyme to possess broad substrate specificity toward lipidic esters if it serves a protective role against oxidative stress since oxidative damage occurs not only from phospholipids but also from various types of compounds containing unsaturated bonds through uncontrolled free radical reactions (2).

The brain enzymes, PAF-AH(Ib) \(\alpha 1/\alpha 1\) and \(\alpha 2/\alpha 2\) have greater preference and selectivity toward an acetyl group than do the other PAF-AHs. PAF-AH(Ib) hydrolyzes an acetyl group not only from PAF and PAF analogues but also from other types of acetyl-containing esters such as octyl acetate or triacetylglycerol. Our results are consistent with a previous study showing strict substrate specificity of the enzyme for an acetyl at the sn-2 position of a phospholipid (18). It has been proposed that PAF in the brain plays an important role as a messenger in excitatory neurotransmitter release, neuronal plasticity, memory formation, and long-term potentiation (reviewed in ref 44). It was also postulated that 1-O-alkyl-2-acetyl-phospholipids other than PAF such as alk-1-enyl-acetyl-glycerylphosphoethanolamine (2-acetylplasmalogen) may be physiological substrates for PAF-AH(Ib) (45). A crystallographic study of PAF-AH(Ib) \(\alpha 1/\alpha 1\) homodimers reveals that the active site structure can account for the exclusion of phospholipids with sn-2 acyl chains longer than acetyl (46).

Like pPAF-AH and PAF-AH(II), physarum PAF-AH displays broad substrate specificity, which raises the possibility that the function of this enzyme includes the hydrolysis of other hydrophobic esters other than PAF in lower eukaryotes. As with PAF-AH(II) and pPAF-AH, it has been speculated that physarum PAF-AH has an anti-oxidative role in the organism through its hydrolysis of oxidatively truncated phospholipids (24).
Properties of Lipoprotein-Bound pPAF-AH. The specific activity of the following forms of pPAF-AH were found to be similar (less than 1.5-fold variation) when enzyme activity was assayed with the substrate [3H]C16-PAF at 100 μM: HDL- and LDL-bound endogenous pPAF-AH, and recombinant pPAF-AH added to LDL or free in solution in the presence of CHAPS (25). Likewise, specific activities (less than 2-fold variation) were seen with these forms of pPAF-AH when enzyme was assayed with 1.1 mM [3H]C10-PAF. Thus, binding of pPAF-AH to HDL or LDL does not significantly change the V_{\text{max,app}} of this enzyme. In our previous study (25), we found that the specific activity of the recombinant pPAF-AH at 21 °C in the absence of vesicles, bound to zwitterionic phosphatidylcholine vesicles, and bound to anionic phosphatidylmethanol vesicles were similar (27, 15, and 16 s^{-1}, respectively) in the presence of saturating PAF, and (54, 48, and 61 s^{-1}, respectively) in the presence of saturating C14-PAF. At 37 °C, the specific activity for C16-PAF in the absence of the vesicles, was found to be 97 ± 11 s^{-1}. Again, these results show that the V_{\text{max,app}} for pPAF-AH is not significantly altered by binding of the enzyme to a phospholipid interface.

In a previous study, it was suggested that the catalytic properties of pPAF-AH are influenced by its lipoprotein environment (8). It was shown that removal of LDL from human plasma by density gradient ultracentrifugation resulted in a 2.3- to 2.8-fold increase in the half-time for the degradation of PAF when added exogenously to plasma in the 10^{-9}–10^{-7} M range. This increase in PAF hydrolysis half-time is consistent with the 2.7-fold decrease in total pPAF-AH activity measured under V_{\text{max,app}} conditions (8). This result is consistent with the fact that the majority of pPAF-AH is bound to LDL (19, Figure 2). Interestingly, when HDL was depleted from plasma, again by density gradient ultracentrifugation, the half-time for exogenous PAF actually decreased by 1.4- to 1.9-fold, despite the fact that the total pPAF-AH activity decreased by 1.5-fold (8). A possible explanation for this seemingly paradoxical result can be found in terms of the aqueous phase model for pPAF-AH action (25). It is known that addition of a physiological concentration of serum albumin to albumin-free LDL containing bound PAF leads to a redistribution of most of the PAF to the albumin fraction (8). It is well established that the HDL fraction obtained from single density gradient ultracentrifugation of plasma contains significantly more albumin than does the LDL fraction. Thus, HDL-depleted plasma, prepared by this method, contains significantly less albumin than does nonfractionated plasma. This in turn suggests that the aqueous phase concentration of PAF is higher in HDL-depleted plasma than in whole plasma. This would result in a decrease in the half-time of PAF hydrolysis when exogenous PAF is added in the 10^{-9}–10^{-7} M range, i.e., under nonsaturating conditions for pPAF-AH, because this enzyme is sensitive to the aqueous phase concentration of PAF. The aqueous phase model also accounts for the observation that the rate of hydrolysis of exogenously added PAF is slightly stimulated in cell-free plasma (8). Again, the cells provide a hydrophobic interface for PAF partitioning, leading to a decrease in aqueous phase PAF concentration. The approximately 2-fold decrease in the half-time for the hydrolysis of exogenously added PAF in serum from a Tangier patient, which lacks HDL, is consistent with the approximately 2-fold higher levels of pPAF-AH measured in the Tangier patient as compared to that measured in plasma from normal patients (47). All together, the data with HDL and LDL depleted plasma do not provide evidence that the catalytic properties of pPAF-AH are influenced by its lipoprotein environment. Indeed, data in the present study argues that the V_{\text{max,app}} of lipoprotein-free and -bound pPAF-AH are very similar. Our studies do not address whether the K_{m} for PAF hydrolysis by pPAF-AH is influenced by the lipoprotein environment. The difficulty with this issue is that values of K_{m} measured for pPAF-AH are apparent values (25), and values of K_{m,app} vary with changes in the concentration of all components in plasma, such as albumin, that can bind to PAF and lower its aqueous phase concentration.

There have been postulates that other isoforms of pPAF-AH with different kinetic characteristics may exist in dense LDL fractions (9). However, our data show that the ratio of total pPAF-AH activity to total PAF-AH protein detected by a monoclonal antibody prepared against recombinant pPAF-AH was the same across all lipoprotein fractions. Thus, there is no indication for the existence of multiple pPAF-AH enzymes, and a single pPAF-AH is also supported by the study of Yoshida et al. which showed that the PAF-AH-deficient patient due to a single mutation in the pPAF-AH gene lacked the PAF degrading activity in the plasma (42).

ACKNOWLEDGMENT

The authors are grateful to Susan Pederson (ICOS) for the development of the quantitative EIA of pPAF-AH, to Matthew Taylor (ICOS) for technical assistance, and to Dr. H. Stewart Hendrickson, Elizabeth K. Hendrickson, and Dr. John Glomset (University of Washington) for the generous gifts of lipids. We also thank Dr. Kohei Yokoyama (University of Washington) for helpful comments on the manuscript.

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