Enhanced Association of Platelet-activating Factor Acetylhydrolase with Lipoprotein (a) in Comparison with Low Density Lipoprotein*

(Received for publication, September 13, 1995)

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Paired samples of human Lipoprotein (a) (Lp(a)) and low density lipoprotein (LDL) were assayed for their platelet-activating factor (PAF) acetylhydrolase activity. Lp(a) displayed markedly enhanced PAF acetylhydrolase activity (approximately 7-fold based on equal particle concentrations) in comparison to LDL isolated from the same individual. Lp(a)-associated acetylhydrolase exhibited properties observed for LDL-associated acetylhydrolase as well as for the purified enzyme; significant inhibition was obtained by treatment with diisopropylfluorophosphate (1 mM, 90%) and phenylmethanesulfonlfuoride. PAF acetylhydrolase activity was demonstrated (9), features that provide evidence for elevated Lp(a) levels as a substantial risk factor. Moreover, an overexpression of apo(a) promotes the development of lesions in transgenic animals (10). Although Lp(a) has also been suggested to interfere in fibrinolysis (11, 12), the actual proatherosclerotic mechanisms remain unknown. Amidolytic activity was found to be associated with apo(a) some time ago (13). More recently, it has been reported that apo(a) contains a trypsin-like protease domain (14) and is capable of catalyzing the cleavage of fibronectin (15).

LDL as well as high density lipoprotein contains an associated acetylhydrolase activity characterized as lipoprotein-associated platelet-activating factor-acetylhydrolase (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) acetylhydrolase (PAF-AH) (16–19). This unique type of enzyme utilizes a serine residue to catalytically cleave phospholipid ester bonds and is also likely to control the PAF concentration in human plasma (20). The enzyme also exhibits a marked selectivity for phospholipids with a short acyl group (21). Hence, the enzyme is capable of hydrolyzing compounds generated by lipid peroxidation (22), which abolishes the PAF-like activity of these substances (23). In conjunction with hydrolytic activity, it has become increasingly apparent that the platelet-activating factor acetylhydrolase exhibits a protective role in terms of oxidative LDL-modification (24, 25) and cell-cell interactions induced by minimally modified LDL (26). Furthermore, an anti-inflammatory effect could be demonstrated in animal experiments (20). These results are in line with our previous data concerning the uptake of oxidatively modified LDL by macrophages, a process found to be affected by the susceptibility of oxidizable phospholipids to undergo enzymatic hydrolysis (27). Uncontrolled receptor-mediated uptake leading to the accumulation of lipids in macrophages and to their conversion to foam cells, however, is considered to be a critical feature of atherosclerosis (28). It follows that the level of the hydrolytic activity of PAF-AH could be one parameter determining the atherogenic potential of oxidizable lipoproteins. In this context, it is of interest that we most recently described that Lp(a) contains a phospholipase A2 activity (29); however, to our present knowledge nothing is known about a PAF-hydrolyzing activity of Lp(a).

* This study was supported by Grants S-7103 (to A. H.) and S-7104 (to G. M. K.) from the Austrian Research Foundation and by the Deutsche Forschungsgemeinschaft (DE 375/2-1; to H. P. D.). This work was presented at the 5th International Congress on Platelet-activating Factor and Related Lipid Mediators, Berlin, Sept. 12–16, 1995. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. This study is dedicated to Prof. R. Neidlein, Heidelberg, on the occasion of his 85th birthday. To whom correspondence should be addressed. Tel.: 49-6221-563447; Fax: 49-6221-563430.

1 The abbreviations used are Lp(a), lipoprotein (a); apoB, apolipoprotein B-100; apo(a), apolipoprotein (a); DPP, diisopropylfluorophosphatase; DTT, dithiothreitol; HPTLC, high performance thin layer chromatography; LDL, low density lipoprotein; N-acetyl PAF, 1-O-hexadecyl-2-desoxy-2-aminoethyl-sn-glycero-3-phosphocholine; PAF, platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine); PAF-AH, platelet-activating factor acetylhydrolase; paraoxon, diethyl-p-nitrophenylphosphate; PMSF, phenylmethanesulfonlfuoride.
acetylated activity of Lp(a), and the properties were compared with those of the LDL-associated enzyme.

**EXPERIMENTAL PROCEDURES**

Materials—1H-labeled PAF was a product of DuPont NEN. 1-Palmityl-sn-2-glycerol-[5,6,8,9,11,12,14,15-1H]arachidonyl-3-phospho choline (45 mBq/mM) was prepared as described (27). BCA reagent with albumin as standard was obtained from Pierce Chemical Co. Econopac columns packed with Bio-Gel P-6DGel and disposable chromatography columns (Bio-Spin) were from Bio-Rad (München, Germany). Silica gel (grade 60, 70–230 mesh) for column chromatography was from E. Merck (Darmstadt, Germany); HPTLC plates (0.25 mm) and TLC plates (silica gel 60, precoated, 20 × 20 cm; layer thickness, 1 mm) were from E. Merck (Darmstadt, Germany) and were pre-eluted with methanol. Cholesterol was measured enzymatically with reagents from Boehringer Mannheim GmbH (Mannheim, Germany). Coomassie Blue R250 and bromophenol blue were purchased from Serva (Heidelberg, Germany); raiin for anion exchange chromatography (DE 52) was from Whatman (Maidstone, UK); -glucosidase was obtained from Boehringer Mannheim, and neuraminidase was from New England Biolabs GmbH (Schwalbach, Germany). The serine containing peptide (peptide 1, LK-Mannheim, and neuraminidase was from New England Biolabs GmbH (Maidstone, UK); and bromphenol blue were purchased from Serva (Heidelberg, Germany), and hexadecyl-2-desoxy-2-amino-oxy-2-aminoacetyl-sn-glycero-3-phosphocholine and subsequent acetylation according to procedures described previously (30, 31); identity was confirmed by comparison with reported experimental data (32). All other chemicals and biochemicals were purchased from Aldrich Chemical Co. (Steinheim, Germany), Fluka (Neu-Ulm, Germany), or Sigma Chemical Co. (Deisenhofen, Germany).

**Analytical Procedures**—After thin layer chromatography, the percentage of PAF hydrolysis was determined with a two-dimensional TLC scanner (Berthold Digital Autoradiograph, Berthold, Wildbad, Germany); proton nuclear magnetic resonance spectra were obtained on a Bruker WM 300 spectrometer (Bruker Physik AG, Karlsruhe, Germany), and proton chemical shifts are relative to tetramethylsilane as internal standard. The solvent gave 540 mg (1.4 mM) of 6-hexyl-(6-O-butyl-(4-pyrenyl))benzoic sulfonamide fluoride (2) by reaction with 290 mg (1.3 mM) 4-(fluorosulfonyl)benzoyl chloride (2 h, 40°C) in the presence of protein A-Sepharose; 1H-NMR (CDCl3): δ 2.30–2.50 (3H, CH3), 3.18–3.31 (4H, -CH2-), 4.3–4.5 (2H, -CO-), 7.85–8.4 (9H, -pyrenyl); 13C-NMR (CDCl3): δ 45.7 (O-CO), 55.9 (aryl-CH2-), 32.97 (aryl-CH2-), 51.77 (O-CO-), 129.26 (aryl-C), 138.57 (4H, -C=O), 151.66 (aryl-C). 1H-NMR (CDCl3): δ 1.25 (4H, -CH2-), 1.7–2.0 (4H, CH2-CH2-), 3.3–3.6 (4H, CH2-(CH2)3-CH2-), 4.4–4.7 (2H, -O-CH2O-), 6.4–7.5 (9H, -pyrenyl); 13C-NMR (CDCl3): δ 27.87, 28.34, 28.47, 29.65 (-CH2-). 1H-NMR (CDCl3): δ 1.25 (4H, -CH2-), 1.7–2.0 (4H, CH2-CH2-), 3.3–3.6 (4H, CH2-(CH2)3-CH2-), 4.4–4.7 (2H, -O-CH2O-), 6.4–7.5 (9H, -pyrenyl); 13C-NMR (CDCl3): δ 27.87, 28.34, 28.47, 29.65 (-CH2-). 1H-NMR (CDCl3): δ 1.25 (4H, -CH2-), 1.7–2.0 (4H, CH2-CH2-), 3.3–3.6 (4H, CH2-(CH2)3-CH2-), 4.4–4.7 (2H, -O-CH2O-), 6.4–7.5 (9H, -pyrenyl); 13C-NMR (CDCl3): δ 27.87, 28.34, 28.47, 29.65 (-CH2-).

4.5(2H,m,-C2H2)4-CH2-), 32.97 (aryl-CH2-), 4.3–4.5 (2H, -CO-), 7.85–8.4 (9H, -pyrenyl); 13C-NMR (CDCl3): δ 45.7 (O-CO), 55.9 (aryl-CH2-), 32.97 (aryl-CH2-), 51.77 (O-CO-), 129.26 (aryl-C), 138.57 (4H, -C=O), 151.66 (aryl-C).
(7:7:2:4, v/v/v/v); furthermore, a 100-μl aliquot of the reaction mixture was extracted with toluene or methylene chloride (2 M; 37°C); aliquots (30 μg) were taken at the times indicated, the reactions were terminated by addition of EDTA (final concentration, 2 mM); and the enzyme activity as measured as described; average values of five paired lipoprotein samples (measured in triplicate) ± S.D. are given.

Inactivation of PAF-AH by 4-Hexyl-(6-O-butyl-(4’-pyrenyl))-benzoc Ester Sulfonyl Fluoride (2)—The kinetics of PAF-AH inactivation were determined using lipoproteins (0.5 ml, 120 μg/ml Lp(a) or LDL) or partially purified enzyme (0.5-ml samples, 0.12 μg/ml) approximately 1340-fold purified from human plasma according to Ref. 17 up to the DEAE-column step, the preparation produced 687 μmol of lyso-PAF/min/mg as a source of enzymatic activity. The reaction (at 37°C) was started by the addition of 2 (from a 100 mM stock solution in Me2SO) to the enzyme in incubation buffer. At selected times, aliquots were removed and diluted 20–80-fold (increasing with inhibitor concentration) into an assay mixture, and residual activity was assayed as described above.

Chemical Nature of Inactivation Process—The irreversibility of inhibition was tested in two ways. First, the inactivated enzyme (partially purified PAF-AH, 60 ng or LDL, 30 μg) was separated from excess inhibitor by dialysis (incubation buffer for 24 h at 4°C with three changes using 2000 times the sample volume each time), and the sample was assayed for return of activity. Second, the sample was subjected to anion-exchange chromatography (1.5 ml of Whatman DE 52 equilibrated with the above buffer in disposable mini columns (Bio-Rad)). The column was washed with 8 ml of dialysis buffer, and the sample (active enzyme as determined in control experiments) was then eluted with 2 ml of the same buffer containing 0.25 M NaCl. After dialysis and concentration, the sample was assayed for activity. Neither procedure restored enzymatic activity.

Determination of Protein-bound Fluorescence after Reaction with 4-Hexyl-(6-O-butyl-(4’-pyrenyl))-benzoc Ester Sulfonyl Fluoride (2)—To estimate the amount of lipoprotein-associated PAF acetylhydrolase, a sample (0.5 ml, 120 μg/ml) of Lp(a) or LDL was incubated with compound 2 (final concentration 450 μM) in Tris-HCl incubation buffer for 3 h at 37°C. The solution was dialyzed against the above Tris-buffer, and the excess reagent was removed (and the apoproteins precipitated) by treatment with precooled acetone (5 ml at –20°C). The mixture was kept at –20°C, and after 1 h, the precipitate was isolated by centrifugation and resuspended in another 5 ml of solvent. The residue obtained after the third washing was dissolved in 250 μl of NaOH (50%, w/v) and kept at 40°C for 24 h. After cooling the solution was neutralized with HCl (final volume, 500 μl) and extracted with toluene or methylene chloride (2 × 250 μl), and the organic phase was separated by brief centrifugation. Fluorescence in the residual aqueous phase as fluorescence in further extracts (>2) was below the detection limit. The extract was dried (Na2SO4) and concentrated to a volume of 100 μl in a stream of nitrogen, and the fluorescence of a 50-μl aliquot measured (methylene chloride, λex 344 nm, λem 398 nm as found for λmax of 1); data obtained from lipoproteins are compared on the basis of relative fluorescence intensities of samples with equal protein concentrations and are corrected by subtraction of fluorescence values obtained for residual unspecific binding as determined by incubation of 2 with DFP-pretreated lipoproteins; approximately 6% of the protein-associated fluorescence (background) was left after DFP-pretreatment (conditions as described for inactivation of the antisera, treatment with 2 as outlined above). In control experiments, radiolabeled lipoproteins were subjected to the above protocol, and 79 ± 14% (LDL, mean of 4 determinations) and 84 ± 18% (Lp(a), mean of 3 determinations) of the radioactivity was recovered in the pellet after the third acetone washing. Because the N-bromosuccinimide method (35) used for the labeling of the lipoproteins affords a maximum of 18% of lipoprotein-bound radioiodine associated with the lipids, these data indicate that the protein fraction of the samples is precipitated completely and is retained almost quantitatively in the pellet. In further experiments, LDL samples labeled with 2 (5 ml, 120 μg/ml protein) or samples of plain compound 2 in buffer were processed as per the procedure described above (acetone extraction of the lipoprotein samples only). HPTLC analysis (hexane/ethyl acetate (3:1, v/v)) including authentic standard and fluorescence spectroscopy confirmed complete conversion of protein-bound (or free) compound 2 to the alcohol 1 and the absence of other fluorescent compounds.

RESULTS

PAF-AH Activity of Lp(a) and Susceptibility to Oxidative Inactivation—We assayed the PAF acetylhydrolase activity of paired Lp(a) and LDL samples isolated from human plasma in all samples we measured consistently significantly elevated levels of lipoprotein (a)-associated hydrolytic activity. Results based on equimolar concentrations of lipoprotein particles are presented in Fig. 1 and indicate that the Lp(a)-associated acetylhydrolase activity exceeded those found associated with LDL 6.9-fold. Analysis of the data (8.9 nm (lyso-PAF)/min × mg protein for Lp(a) and 2.2 nm/min × mg for LDL) on the basis of equal protein concentrations affords a ratio of the activities of 4 (Lp(a)):1 (LDL).

The LDL-associated PAF acetylhydrolase is sensitive to Cu2+-initiated lipid peroxidation, a process that is accompanied by a concomitant decrease of enzymatic activity (36). To determine the relative sensitivity of the Lp(a) associated enzyme, the time-course was analyzed under pro-oxidative conditions (Fig. 1). We found that the Lp(a)-associated activity decreased somewhat faster than the LDL-associated activity.
Although 50% of activity was left in Lp(a) after 8 h, the LDL-associated activity was reduced to 57%. After 15 h, Lp(a) displayed 9% of the initial activity, whereas LDL contained 19% of the basal activity. The relative decrease in hydrolytic activity of Lp(a) was slightly enhanced under pro-oxidative conditions; the absolute activity, however, exceeded the LDL-associated activity at all times monitored.

Inhibition of Lp(a)-associated PAF Acetylhydrolase Activity—To characterize the properties of the enzymatic activity of Lp(a) in comparison to LDL, we used DFP and PMSF, which are known inhibitors of the plasma enzyme (37, 38). As shown in Fig. 2, substantial inhibition (reduction by 90%) of both samples was achieved by incubation with 1 mM DFP for 2 h. A parallel reduction of the hydrolytic capacity was observed after treatment with 5 mM PMSF (60% for LDL and 52% for Lp(a)) and with the cholinesterase inhibitor paraoxon (1 mM) (49). We found that paraoxon reduced the activity of both particles equally in a concentration-dependent manner (Fig. 2, inset) with an IC_{50} of approximately 0.9 mM. At a concentration of 6 mM, complete inhibition was observed in both samples. As reported for the LDL associated enzyme (38, 39), PAF hydrolysis by Lp(a) could not be reduced by treatment with p-bromophenacylbromide and was insensitive to DTT at a concentration of up to 100 mM. In agreement with a previous report (38), C_{1}-palmitoyl-2-[7-nitrobenez-2-oxa-1,3-diazol-4-yl]-amino]hexanoyl-sn-glycero-3-phosphocholine was found to be a substrate of the Lp(a) associated activity. As expected, the Lp(a) associated activity failed to cleave the ester bond of 1-palmitoyl-sn-2-glycero-[5,6,8,9,11,12,14,15-3H]-arachidonoyl-3-phosphocholine, indicating a preference for substrates with short acyl chains as reported for the isolated protein (20) (data not shown). Hence, the hydrolytic activity of Lp(a) displayed all characteristics known for the LDL-associated enzyme as well as for the purified PAF acetylhydrolase, indicating that this enzyme is the sole source of the Lp(a)-associated PAF hydrolytic activity.

Effects of Glycolytic Enzymes—Because apo(a) is a highly glycosylated protein (3), we examined whether treatment with glucosidases could affect either enzymatic activity or association of the enzyme to Lp(a). Incubation (4 h) of Lp(a) (120 µg/ml) with a combination of β-glucosidase (200 units/ml) and neuraminidase (100 units/ml) caused a loss of 88% of sialic acid (as determined from apoLp(a) by the thiobarbituric acid procedure according to Ref. 40) and a loss of 47% of total carbohydrate content (determined from apoLp(a) according to Ref. 41, average values of two experiments). However, only 20% of enzymatic activity (relative to an untreated control as determined after reisolation by gel filtration chromatography or by native agarose electrophoresis, data not shown) was lost suggesting that glycosylation is not critical to enzyme-apoprotein interactions, to the access of substrate and/or to the release of products from the active site.

Apoprotein Association of PAF Acetylhydrolase Activity—To probe the association of the enzyme to apo-Lp(a) we used antibodies for immunoprecipitation. Treatment of Lp(a) with rabbit or sheep anti-apo(a)-antiserum and subsequent centrifugation resulted in a significant loss of enzymatic activity (8 and 15% residual activity, respectively, Table I). Because apo(a) can be dissociated from apoB under reducing conditions (42), we investigated the enzyme-apoprotein association after disulfide reduction. Lp(a) was treated with DTT, and apo(a) was precipitated by use of the anti-sera. A substantial portion of PAF acetylhydrolase activity (67 and 74% of control activity, respectively) remained in the supernatant. Incubation and precipitation with anti-apoB antibodies, however, removed the activity almost completely, a result that was unaffected by a preceding DTT treatment. The loss of acetylhydrolase activity found in DTT-pretreated Lp(a) after incubation with anti-apo(a) antisera may be explained by partial coprecipitation of apoB with apo(a) immunocomplexes due to noncovalent interactions.

Characterization of the Fluorescent Probe 4-Hexyl-(6-O-buty1-(4'-pyrenyl))-benzoic Ester Sulfonyl Fluoride (2)—The novel fluorescent agent 4-hexyl-(6-O-buty1-(4'-pyrenyl))-benzoic ester sulfonyl fluoride (2) (Fig. 3) was designed to achieve nonradioactive labeling of PAF-AH, allowing the determination of enzyme bound compound or vice versa, the quantitation of enzyme. We reasoned that the poor inhibitory potency of the PAF-AH inhibitor PMSF may be improved by the introduction of an aliphatic chain attached to the phenyl group carrying the reactive methylsulfonyl fluoride moiety. This variation promised an increase of the structural resemblance to the original substrate PAF. Moreover, the reactivity of the sulfonfluoride with nucleophilic amino acid residues should be enhanced by a direct phenyl-sulfonyl fluoride linkage. We therefore synthesized the pyrene-labeled compound 2 and characterized its properties and in particular its effects on lipoprotein associated as well as on partially purified PAF acetylhydrolase. First, the behavior in the absence of PAF...
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Reduction of PAF acetylhydrolase activity by immunoprecipitation of apoproteins

Residual PAF acetylhydrolase activity after treatment with anti-apoB and anti-apo(a) antibodies. Lipoproteins (0.12 mg/ml) were incubated with limiting amounts of antibodies to apo(a) and apoB (16 h at 4°C) followed by precipitation with protein A-Sepharose. The remaining activity is given relative to an equally treated (except for the antibodies) control sample. Antisera were inactivated with DFP as described under "Experimental Procedures." Except for sample size, conditions of PAF-AH activity determination were as described in the legend to Fig. 1.

\[ \text{PAF-AH activity} \]

|        | LDL | Lp(a) |
|--------|-----|-------|
| Anti-apoB | <5  | <5    |
| Anti-apo(a) | 8 (15) | 67 (74) |

\(^a\) A 10 mM DTT solution was used (2 h at 37°C) for reductive disulfide cleavage. The binding of the antibodies as the immunoprecipitation of the lipoproteins was unaffected by this DTT concentration as observed in control experiments where the reagent was removed by dialysis prior to addition of the antisera.

\(^b\) Immunoprecipitation conducted with anti-human apo(a) polyclonal antiserum from rabbit or from sheep (values in parentheses). The data are representative of two separate experiments.

![Structure of the fluorescent inhibitor 4-hexyl-(6-O-butyryl-(4'-pyrenyl))-benzoic estersulfonylfluoride (2).](Image)  

The activity corresponds to \( \varepsilon = E_0 - [E-I] \) \( E_0 = [E] + [E+I] + [E-I] \) and \( [E-I] \) = concentration of the modified/inactivated enzyme. The solution of the equation \( -d[E]/dt = k_2 [E+I] \) then affords Eq. 2 \( \varepsilon = (ln \varepsilon + a)/t \) (44). The value of \( k_{obs} \) was determined directly from the slope of the lines in Fig. 4. A double reciprocal plot of the slope of each line \( k_{obs} \) versus inhibitor concentration \( I \) according to Equation 3.

\[ \frac{1}{k_{obs}} = K_{inact}/k_2[I] + 1/k_2 \] (Eq. 3)

(Fig. 4, inset) provided the \( K_{inact} \) value = 525 \( \mu M \) and the \( k_2 \) value 0.03 min\(^{-1}\).

Consistent with the notion that covalent modification of the hydrolase has taken place, extensive dialysis of the inhibitor-enzyme reaction mixture as anion-exchange chromatography failed to restore enzymatic activity.

Correlation between the Content of Active PAF Acetylhydrolase and Protein-bound Fluorescence—Given a defined ratio between active PAF acetylhydrolase and protein-bound fluorescence after inactivation with compound 2, the fluorescent probe should enable the comparison of the amounts of LDL- and Lp(a)-associated enzyme. To analyze the relationship between protein-bound fluorescence and the relative amount of active enzyme, Lp(a) samples were inactivated with DFP and recombined with untreated fractions of the same lipoprotein. The combined samples then were treated with compound 2 at conditions providing complete inactivation of residual enzymatic activity, and protein-bound fluorescence was determined (Fig. 5). A linear relation \( (r^2 = 0.98) \) was observed between the fraction of enzymatically active lipoprotein and the fluorescence associated with the apoprotein. Similar data were obtained in analogous experiments with recombinant LDL samples \( (r^2 = 0.96) \), agreement of data points with the presented values of Lp(a) within 19% when samples with the same enzymatic activity prior to treatment with 2 were used) as well as in experiments with partially inactivated purified enzyme \( (r^2 = 0.98) \), data not shown). These results indicate that the analysis of
protein-bound fluorescence can be utilized as a measure of the amount of active enzyme. Consistently, pretreatment of Lp(a) with DFP to complete enzymatic inactivation precludes fluorescence labeling almost completely (Fig. 5, inset), suggesting that both agents occupy the same enzymic site. Moreover, the presence of the unhydrolyzable PAF analog N-acetyl-PAF (K
\text{rev}
 with purified PAF-AH about 370 
\mu
m
) during treatment of Lp(a) with inhibitor 2; 1, fluorescence extracted from Lp(a)-protein; 2, fluorescence extracted from LDL protein after labeling of the lipoproteins (paired samples, both 120 
\mu
m
 with inhibitor 2; 3, fluorescence extracted from Lp(a) protein (background fluorescence) after complete DFP inactivation of Lp(a) prior to the addition of 2. The ratio of values \( \frac{1}{2} = 3.4:1 \). Mean values of experiments performed in triplicate are given, representative of two separate experiments.

Comparison of Enzyme Content of LDL and Lp(a)—The increased level of enzymatic activity found with Lp(a) could be a reflection of the binding of a greater amount of enzyme. Inactivation of lipoprotein-associated enzyme with compound 2 was carried out to estimate the relative content of active enzyme in both lipoproteins. Using equal protein concentrations, we compared protein-bound fluorescence measured upon treatment of both lipoproteins, LDL and Lp(a), with the sulfonyl fluoride (2). We found that Lp(a) (the apoproteic part) released 3.4-times more fluorescent compound 1 (Fig. 5, inset), a value that compares favorably with the ratio (4:1) of the lipoprotein-associated PAF-hydrolyzing activities.

**DISCUSSION**

In the experiments presented we have characterized the Lp(a)-associated PAF acetylhydrolase activity and studied the differences to the LDL-associated one. We demonstrate for the first time that Lp(a) exhibits several fold enhanced PAF acetylhydrolase activity compared with LDL. Data analyzed on a molar basis show that the Lp(a)-associated activity exceeds the LDL-associated activity approximately 7-fold. The enzyme associated with Lp(a) exhibits a number of known properties like substrate specificity and sensitivity to different inhibitors found in the LDL-associated PAF acetylhydrolase as well as in the purified protein. These results confirm that the Lp(a) associated PAF acetylhydrolase is identical to the enzyme found associated with LDL.

Because Lp(a)-associated PAF acetylhydrolase is likely to affect oxidative modification of the Lp(a) particle, we determined its susceptibility to oxidative inactivation in comparison to the LDL-associated activity. We found that despite Lp(a)'s greater stability toward oxidation (45), the relative decrease in hydrolytic activity of Lp(a) was slightly enhanced under prooxidative conditions. This suggests that the enzyme, when it is associated with Lp(a), may be more susceptible to oxidative damage than the enzyme associated with LDL. The absolute activity (per particle or per mg protein), however, exceeded the activity shown by the LDL-associated enzyme at all times monitored.

Considering the observations outlined above, it was of particular interest to determine the basis for the differences between lipoprotein-associated activity found with LDL and with Lp(a). Therefore, the fluorescent compound 2 was prepared as a tool to estimate the quantity of lipoprotein-associated enzyme and to analyze the reason for the difference in hydrolytic abilities observed between LDL and Lp(a). This compound irreversibly inhibits lipoprotein-associated or partially purified PAF acetylhydrolase, and the observed progressive development of inhibition is consistent with the hypothesis that it reacts with the enzyme (possibly with the likely active site nucleophile serine 273) (20) to yield a sulfonyl enzyme derivative. Kinetics of 2 is typical for an irreversible inhibitor (44), and the amount of fluorescent compound bound to the protein fractions of the lipoproteins after treatment with 2 is linearly related to the content of active enzyme. This was evident when samples of partially inactivated lipoproteins were combined with enzymatically active fractions, and the protein-bound fluorescence of the mixture was determined. We further found that similar results were also obtained when samples of different classes of partially inactivated lipoproteins (LDL and Lp(a)) were combined (enzymatic activities were found to be additive) and subsequently inactivated with the fluorescent probe 2. Therefore, in the micellar assay system, the kinetics of substrate hydrolysis as well as the reaction with the sulfonyl fluoride (2) appears to be predominantly independent of the presence of other lipoprotein components.

Several experiments provide evidence that fluorescence labeling with 2 is active site-specific. Furthermore, the line obtained by plotting the values of \( k_{\text{obs}} \) against Equation 3 did not pass through the origin but intercepted the positive y axis indicating the initial formation of reversible complexes. Our data further indicate incorporation of fluorescent label into the enzymatically active enzyme at a defined and constant ratio (label/mol enzyme), thus demonstrating the fulfillment of requirements for enzyme quantitation. The sulfonyl fluoride (2) of related compounds may represent sensitive tools to label and quantitate PAF acetyldiolase, and further structural variations promise compounds with increased inhibitory potency.

The lipoprotein environment alters the catalytic behavior of PAF-AH (46). However, our results suggest that the increased hydrolytic activity of Lp(a) is primarily due to the binding of a greater number of enzyme molecules. Previous studies from our laboratories provide evidence that Lp(a) displays a de-
creased mobility on the surface (29, 47), a feature that may affect the association of apoproteins (48) or could be due to apo-Lp(a) interactions. Hence, differences found in the association of PAF acetylhydrolase with LDL and Lp(a) could be attributed to variations in apoprotein-lipid interactions affecting the binding of the enzyme and/or might be due to alterations in direct interactions between the enzyme and the lipid surface.

Taken together, our data suggest that PAF acetylhydrolase exhibits an enhanced affinity to Lp(a)-apoB, resulting in an increased enzyme/lipoprotein ratio relative to LDL. It remains to be clarified how the potentially protective role of PAF acetylhydrolase is compatible with the apparent atherogenic properties of Lp(a).
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J. Biol. Chem. 1995, 270:31151-31157.
doi: 10.1074/jbc.270.52.31151

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