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Biochimica et Biophysica Acta 1323 (1997) 12–22

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

The platelet-activating factor acetylhydrolase of mouse platelets

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Received 6 August 1996; accepted 4 September 1996

Abstract

Platelet-activating factor (PAF) acetylhydrolases are a family of distinct enzymes with the common property of hydrolyzing and inactivating PAF. It has been shown that the structure and the biochemical behavior of these enzymes depend on their cellular origin. We studied the PAF acetylhydrolase activity in mouse platelets in order to investigate the unusual response of these platelets to PAF. We found that mouse platelets contain a PAF acetylhydrolase with an apparent K_m value of 0.8 μM, suggesting a very high affinity for PAF. Contrary to other normal mammalian cells and tissues, mouse platelet PAF acetylhydrolase is almost equally distributed in the membranes and the cytosol and is characterized by an extreme sensitivity to heating. The enzyme requires the presence of dithioerythritol for maximal activity, it is affected by 5,5′-dithiobis(2-nitrobenzoic acid) and N-ethylmaleimide, and it is strongly inhibited by phenylmethylsulfonylfluoride. We purified, to near homogeneity, the PAF acetylhydrolase from mouse platelet membranes and demonstrated that it is a protein relatively abundant in the membranes with an apparent molecular weight of 270 kDa. Electrophoretic analysis, under reducing conditions, revealed four bands and one duplet with molecular weights of 66, 55, 52, 49 and 62 kDa, respectively. Thus, PAF hydrolysis in mouse platelets is mediated by a PAF acetylhydrolase having biophysical and biochemical properties more intricate than those of the PAF acetylhydrolases found in other species.

Keywords: Mouse platelet; PAF; PAF acetylhydrolase; Membrane-bound enzyme; Purification

1. Introduction

Platelet-activating factor (PAF,1-0-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a bioactive phospholipid mediator, that appears to participate in pathological processes (e.g., inflammation, allergy, asthma, acute bronchoconstriction) as well as in physiological events (e.g., reproduction, blood pressure) in several species (reviewed in Refs. [1–3]).

PAF in vitro can activate a variety of cells, including platelets, neutrophils and macrophages and is produced upon appropriate stimulation by many types of cells and tissues [4,5]. Its inactivation occurs by hydrolysis of the sn-2 acetyl group of the glycerol backbone. The reaction is enzymatically catalyzed by a PAF acetylhydrolase (PAF-AH) which appears to be the key enzyme for regulating the extracellular and intracellular levels of PAF [6–9].

PAF-AHs have been detected in plasma, blood

Abbreviations: PAF (platelet-activating factor), 1-0-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine; PC, L-α-phosphatidylcholine; EDTA, ethylendiaminetetraacetic acid; DTNB, 5,5′-dithio-bis(2- nitrobenzoic acid); PMSF, phenylmethylsulfonylfluoride; DMSO, dimethyl sulphoxide; DTE, dithioerythritol; NEM, N-ethylmaleimide; DOC-Na, sodium deoxycholate; CHAPS, 3-[(3-cholamidopropyl) dimethilammonio]-1-propane sulfonate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TCA, trichloracetic acid; DTT, dithiothreitol; CMC, critical micellar concentration.

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PII S0005-2736(96)00178-2

cells and tissues of various species and can be secreted by some types of cells (for review, see Ref. [10,11]). They appear to be a multi-component family of enzymes that differ in their biochemical properties even within the same species. PAF-AHs have been purified from human plasma [12] and erythrocytes [13], from bovine brain [14] and more recently, from bovine liver [15]. During the last decade, evidence has accumulated which suggests that PAF-AH mediates a protective mechanism against oxidative damages because, in addition to PAF, the PAF-AHs also utilize, as substrates, toxic oxidatively fragmented phospholipids [16,13–15]. Furthermore, changes in the levels of plasma PAF-AH were observed in various diseases [10]. Recently, Tjolker et al. [17] proposed that PAF-AH might be utilized in the therapy of acute inflammation.

Our interest in mouse platelet PAF-AH originated from the fact that mouse has a peculiar response to PAF. Contrary to other species, mouse is very resistant to PAF when this phospholipid is administered in vivo [18] and moreover, mouse platelets do not respond to PAF with aggregation, even after stimulation by high concentrations (e.g., $5 \cdot 10^{-3}$ M) of this agent [18,19]. We considered that this different behavior of mouse platelets to PAF could coexist with other differences related to the intracellular metabolism of this mediator.

In the present study, we found that mouse platelets contain high levels of PAF-AH, which is almost equally distributed in the cytosolic and the membrane fractions. In order to investigate the role of this enzyme in mouse platelets, we examined some of its biochemical properties and purified, to near homogeneity, the PAF-AH from platelet membranes.

2. Materials and methods

2.1. Materials

1-0-Hexadecyl-2-[^{3}H]acetyl-sn-glycero-3-phosphocholine ([^{3}H]acetyl-PAF, 7.1 Ci/mmol) was purchased from DuPont NEN (Boston, MA, USA). Unlabeled 1-0-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine (PAF), egg yolk L-α-phosphatidylcholine, Phenyl Sepharose CL-4B and CHAPS were from Sigma Chemical Co. (St. Louis, MO, USA). Superose 6B and Sephadex G-200 were from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Hydroxylapatite was from Bio-Rad. All the other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and the solvents from Merck (Darmstadt, Germany), Carlo Erba (Milan, Italy) and Riedel-de Haen (Seelze, Germany).

2.2. Enzyme preparations

Male Swiss SWR mice (20–25 g) were placed in an atmosphere of diethyl ether until respiration ceased. The thoracic cavity was immediately opened and blood (0.8–1 ml) was collected by cardiac puncture through a 0.6 mm-gauge needle into a plastic syringe containing EDTA (0.2 M) in a ratio of 1 part EDTA to 40 parts blood. Blood from four to five mice was pooled and centrifuged at $250 \times g$ for 15 min at room temperature to prepare the platelet-rich plasma (PRP). The platelets were isolated from PRP by centrifugation at $1400 \times g$ for 15 min at 4°C. The pellet was suspended gently in 3 ml of Buffer A (50 mM Tris-HCl (pH 7.4), containing 0.1 M NaCl and 1 mM EDTA). After centrifugation at $1400 \times g$ for 15 min at 4°C, the supernatant was discarded and the pellet was resuspended in Buffer A at a final concentration of $2.0–3.0 \times 10^9$ platelets/ml. The platelet preparation was tested for its ability to hydrolyze exogenously added PAF. The platelets were then sonicated at 4°C for 18×25 s with a 25-s interval using a sonicator (MSE) at an output setting of 4. The resulting lysate, containing less than 1% unbroken platelets, as was calculated by hematocytometric observation, was then centrifuged at $104\,000 \times g$ for 60 min at 4°C to obtain the cytosolic fraction (supernatant) and the total membrane fraction (pellet). The pellet was resuspended in Buffer A and homogenized. Platelet cytosolic and total membrane fractions could be stored at −80°C for at least 4 months without significant loss of enzymatic activity.

2.3. Enzyme assay

Determination of PAF-AH activity was carried out using [^{3}H]acetyl-PAF as substrate. The specific activity of the enzyme was expressed as nmol PAF/h/mg

of protein. The protein concentration was measured by the method of Bradford [20], with bovine serum albumin as the standard.

The incubation mixture consisted of 100 mM Tris-HCl (pH 7.4), 1 mM EDTA, 30 μM [^{3}H]acetyl-PAF (spec. act. 30 000 cpm/nmol) and the enzyme preparation, in a total volume of 200 μl. In assaying the intact platelet PAF-AH activity, 100 μl of their suspension (2×10^8 platelets) were used. In the case of solubilized membranes, the final CHAPS concentration was 0.65 mM.

The kinetic parameters for hydrolysis of sn-2 acetate by platelet lysate, total membrane and cytosolic fractions were determined by varying the substrate concentration between 0 and 40 μM. The effect of various agents on PAF-AH activity, was assayed at a standard concentration of 10 μM PAF.

Incubations were carried out for 30 min at 37°C and the reaction was stopped by adding 0.75 ml of chloroform/methanol (1:2, v/v) according to Bligh and Dyer [21]. For the separation of the two phases the final ratio of the chloroform/methanol/water was adjusted to 1:1:0.9 (v/v) and the [^{3}H]acetate generated by the hydrolysis of PAF was measured in 0.6 ml of the aqueous phase.

2.4. Purification of the PAF-AH from platelet membranes

Solubilization of membranes: Platelet membranes were isolated as described under enzyme preparations and total membranes were obtained by combining the platelet lysates of 50 mice. The total membrane fraction (7.3 mg of protein) suspended in Buffer A (5 ml), was stirred for 60 min at 4°C in the presence of 13 mM final CHAPS concentration (CMC 10 mM). The mixture was then centrifuged at 104 000 $\times g$ for 60 min at 4°C and the supernatant (6.1 mg of solubilized membrane protein) was used for the purification of PAF-AH.

Superose 6B column: The solubilized membranes (5 ml) were applied to a Superose 6B column (1.4 $\times$ 30 cm) and the elution was carried out with Buffer B (10 mM Tris-HCl (pH 7.4) containing 0.1 M NaCl, 1 mM EDTA, 0.5 mM DTE and 0.65 mM CHAPS), at a flow rate of 12.5 ml/h, at 4°C. Fractions (0.7 ml) were collected and assayed for PAF-AH activity and for protein content.

Phenyl Sepharose CL-4B column: The active fractions (15 ml) from the Superose 6B column were pooled and loaded at room temperature on a Phenyl Sepharose CL-4B column (1.25 $\times$ 2.5 cm) which was equilibrated with Buffer C (10 mM Tris-HCl (pH 7.4) containing 0.1 mM EDTA and 0.5 mM DTE), at a flow rate of 6 ml/h. A stepwise elution was carried out at 4°C using increasing concentrations of 3.3, 6.5, 8, 10 and 13 mM CHAPS (6 ml each) in Buffer C. Fractions (0.6 ml) were collected and assayed for PAF-AH activity and for protein content.

Batch Hydroxylapatite: The active fractions (7 ml) from the Phenyl Sepharose CL-4B column were pooled and stirred for 20 min at 4°C with 50 mg hydroxylapatite, previously equilibrated with Buffer D (5 mM potassium phosphate (pH 6.8) containing 10 mM CHAPS). The activity was extracted stepwise from the hydroxylapatite at 4°C, with increasing concentrations of 5, 10, 50, 100, 150 and 200 mM potassium phosphate (0.4 ml each) in Buffer D. The elution time was 15 min for each step. The active fractions (1.8 ml) were pooled and ultrafiltrated using a YM-10 filter (Amicon Corp.)

Native-PAGE: The resulting enzyme preparation after lyophilization was suspended in 50 mM Tris-HCl (pH 6.8) buffer containing 0.3 M sucrose and 0.025% bromophenol blue. The final CHAPS concentration was adjusted to 10 mM. Electrophoresis under non-denaturing conditions was carried out by the method of Davis [22]. Acrylamide solutions were made in 0.7 mM CHAPS and the running buffer in 0.5 mM CHAPS. A 7.5% acrylamide gel was run at a constant voltage of 150 V and then sliced into 0.4-cm horizontal strips. The gel slices were incubated with 10 mM Tris-HCl buffer (pH 7.4) for 36 h at 4°C. The supernatant was assayed for PAF-AH activity.

SDS-PAGE: 3.5 μg of protein extracted from the gel slice corresponding to the peak of activity in the native-PAGE, was precipitated by the DOC-TCA method [23]. Then 20 μl of sample buffer containing 50 mM Tris-HCl (pH 6.8), 0.3 M sucrose, 2% SDS and 0.025% bromophenol blue was added to the precipitate and the mixture was incubated for 10 min at 95°C. SDS-PAGE with an acrylamide concentration of 7.5%, was performed as described by Laemmli [24]. After electrophoresis at a constant voltage of 150 V, proteins were stained with silver nitrate.

In order to detect the existence of enzyme subunits

SDS-PAGE, under reducing conditions, was performed. One μg of the same preparation was precipitated as mentioned above, solubilized in the same sample buffer supplemented with 50 mM DTT and run on a 7.5% acrylamide gel, under the same conditions. Proteins were visualized by silver nitrate staining.

3. Results

3.1. Kinetic studies of mouse platelet PAF-AH

We studied the kinetic parameters of PAF-AH in the platelet lysate, total membrane and cytosolic fractions. Control experiments for PAF-AH activity in intact platelets did not show any detectable PAF hydrolysis. For the lysate, as well as for the total membrane fraction of mouse platelets, PAF hydrolysis was linear up to 7 μg of protein and for an incubation time of up to 45 min. For the cytosolic fraction, PAF hydrolysis was linear for up to at least 30 μg of protein and for an incubation time of up to 60 min. The rate of PAF hydrolysis followed Michaelis Menten kinetics for all three enzyme preparations.

The apparent K_m and V_{max} values were calculated from the Lineweaver-Burk double reciprocal plot. For the lysate, membrane and cytosolic preparations, the apparent K_m values were 0.96 ± 0.12, 0.85 ± 0.22 and 0.83 ± 0.14 μM and the V_{max} values were 62, 88 and 45 nmol/h/mg, respectively (Table 1).

The relative distribution of the total PAF-AH activity, in the total membrane and cytosolic fractions, was found to be 57% and 43%, respectively, as calculated from the V_{max} values and the protein content of the two fractions (Table 1).

3.2. Biochemical properties of mouse platelet PAF-AH

Table 2 summarizes the influence of various compounds on platelet PAF-AH activity in both the cytosolic and the total membrane fractions. In general, no significant differences were observed between the two enzyme preparations.

In order to determine whether PAF-AH has similar properties to PLA_2, agents that influence the activity of typical PLA_2 were added. Ca^{2+} inhibited PAF-AH activity while the divalent metal chelator, EDTA, increased, slightly, PAF hydrolysis. Moreover, the addition of egg phosphatidylcholine, even at 100 μM (i.e., at a 10 times higher PAF concentration), caused a slight inhibition of PAF hydrolysis that is apparently due to the high lipid concentration in the incubation mixture [25].

Platelet PAF-AH was affected by Mg^{2+} to the same extent as Ca^{2+}, while Mn^{2+} was more inhibitory, and heavy metal ions, such as Cd^{2+} and Zn^{2+} at 10 mM, poisoned the enzyme, in accordance to their effect in the human erythrocyte enzyme [26]. NaF, an inhibitor of PLA_2, at 10–20 mM seems to have no significant effect on PAF hydrolysis. It has been reported that the effect of NaF on PAF-AH depends on its cellular origin [27].

We then tested the effect of various agents that inactivate enzymes containing essential sulfhydryl groups. DTNB caused an apparently constant inhibition over a wide range of concentrations (0.1–2 mM) and NEM at 2 mM inhibited PAF hydrolysis to the

Table 1
Kinetic parameters and distribution of PAF acetylhydrolase activity in mouse platelets

Cellular preparation	K_m * (μM)	V_{max} (nmol/h/mg)	Total protein (mg)	Total activity (nmol/h)	Distribution of activity (%)
Lysate	0.96 ± 0.12	62	9.12	565	100
Membranes	0.85 ± 0.22	88	3.64	320	57
Cytosol	0.83 ± 0.14	45	5.48	247	43

Preparations of platelet lysate, membranes and cytosol containing 5, 5 and 10 μg of protein, respectively, were incubated for 30 min at 37°C in the presence of [^{3}H]acetyl-PAF (0–40 μM) as described in Section 2. K_m and V_{max} values were estimated from the Lineweaver-Burk double reciprocal plots. * K_m values are the means $\pm$ SD from 3 independent experiments.

same extent as DTNB at 2 mM, while DTE stimulated PAF-AH. PMSF, a serine hydrolase inhibitor for the cytosolic form of PAF-AH [8], inhibited mouse platelet PAF-AH in a concentration-dependent manner. Finally, the enzymatic activity was strongly affected by anionic detergents such as DOC-Na (CMC 4–8 mM) and SDS (CMC 8.2 mM), at concentrations much lower than their CMC value; however, it was less sensitive to the non ionic detergent Triton X-100 (CMC 0.25 mM).

3.3. Purification of PAF-AH from platelet membranes

The unusually high percentage of PAF-AH activity present in platelet membranes led us to utilize the total membrane fraction of mouse platelets for the subsequent purification of the PAF-AH. Among the various detergents examined for their ability to solubilize platelet membranes and their inhibitory effect on the solubilized enzyme, CHAPS at 13 mM (CMC 10 mM), proved to be the most effective. The yield of solubilized protein was 84% and subsequent dilution (20-fold) of the solubilized preparation was necessary to obtain the optimal conditions for assaying PAF-AH activity. Under these conditions, a 70–75% solubilization of the total activity found in platelet membranes, was recovered.

Due to the small quantity of blood per animal, the purification procedure was performed with a very low initial amount of solubilized protein (6.1 mg). We therefore chose this specific series of column purification steps, in order to avoid multiple dialyses

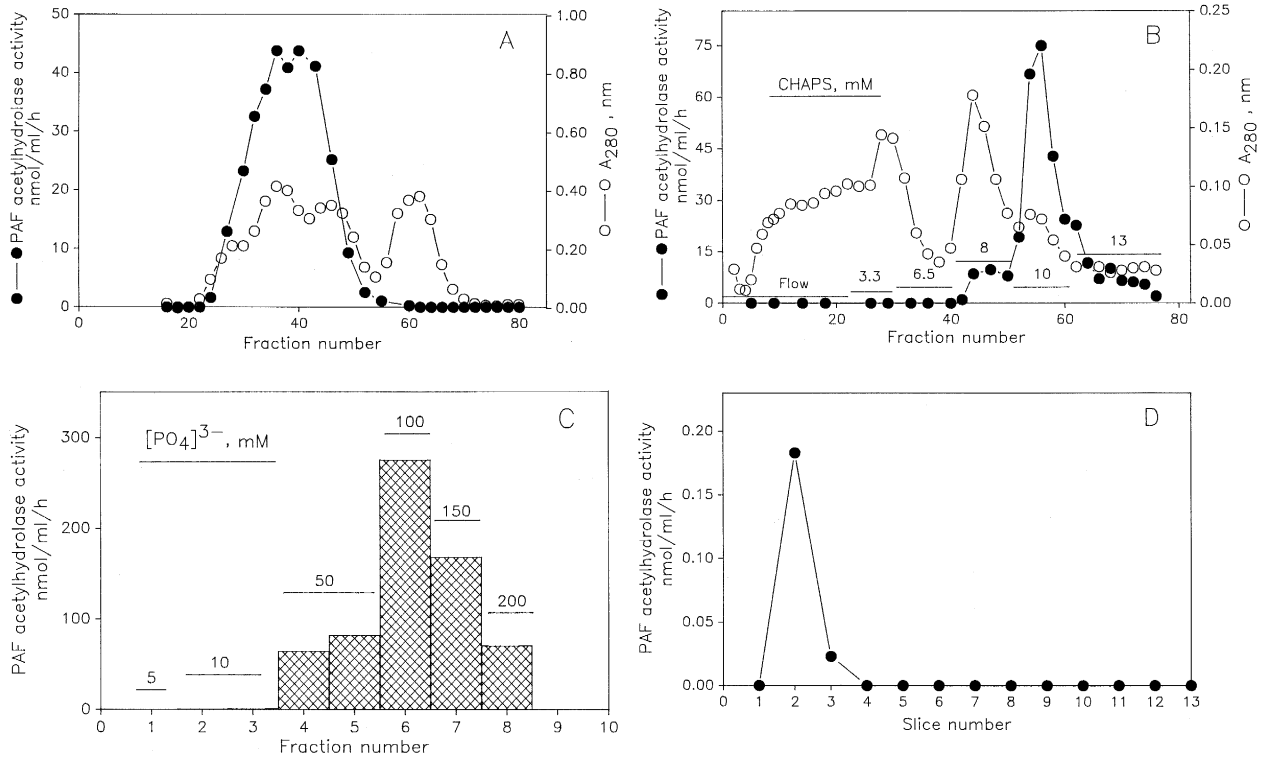

Fig. 1. Purification steps of platelet total membrane fraction PAF acetylhydrolase. (A) Superose 6B. After solubilization with CHAPS, the total membrane fraction (6.1 mg of solubilized protein) was loaded on a Superose 6B column. Fractions (1 ml) were collected and assayed for activity and for protein content. (B) Phenyl Sepharose CL-4B. The active fractions (15 ml) from the Superose 6B column were loaded on a Phenyl Sepharose CL-4B column. Fractions (0.7 ml) were collected and assayed for activity and for protein content. (C) Hydroxylapatite batch. The active fractions (7 ml) from the Phenyl Sepharose column were mixed with 50 mg of Hydroxylapatite and elution was performed stepwise, with increasing concentration of phosphate buffer. Fractions (0.4) were collected and assayed for PAF acetylhydrolase activity. (D) Native-PAGE. The active fractions (1.8 ml) from the Hydroxylapatite were pooled, ultrafiltrated and placed on a gel of 7.5% acrylamide. After electrophoresis the gel was sliced into horizontal strips, which were extracted and assayed for PAF acetylhydrolase activity.

Table 2
Effect of various agents on mouse platelet PAF acetylhydrolase activity

Addition	Concentration (mM)	PAF acetylhydrolase activity (% of control)	
		Membranes	Cytosol
$CaCl_2$	1	82	77
	2	72	ND
	10	62	71
EDTA	1	117	ND
	2	116	ND
	10	113	ND
PC [a]	0.02	99	88
	0.1	86	65
$MgCl_2$	10	66	72
$MnCl_2$	10	48	46
$CdCl_2$	10	7	17
$ZnCl_2$	10	2	2
NaF	10	97	97
	20	88	89
DTNB	0.1	77	83
	0.5	82	85
	1	72	77
	2	59	63
NEM	2	64	69
DTE	5	137	149
PMSF [a]	0.1	34	45
	0.5	17	18
	1	13	14
	2	7	11
DOC-Na	1	74	62
	2	22	23
	4	4	4
SDS	0.35	ND	38
	1.73	ND	4
Triton X-100	0.16	92	90
	1.6	38	ND

Preparations of platelet membranes and cytosol containing 5 and 10 μg of protein, respectively, were preincubated with the various agents for 5 min at 37°C. After addition of [^{3}H]acetyl-PAF at a final concentration of 10 μM, the incubation was continued for 30 min and PAF acetylhydrolase activity was assayed as described in Section 2. Control activity in these experiments was 48 nmol/h/mg for the membranes and 31 nmol/h/mg for the cytosol. Results are the means of two independent experiments performed in duplicate. ND, not determined.[a] PC was dissolved in ethanol and PMSF in DMSO. Any effects due to solvents have been subtracted.

and ultafiltrations, which rendered the solubilized membranes unstable (e.g., at CHAPS concentrations below 0.3 mM, a protein precipitate was observed).

Fig. 1 shows the purification procedure of the PAF-AH from the platelet membranes. The solubilized membrane fraction was first loaded onto a Superose 6B gel filtration column (Fig. 1A). The active peak fractions from the size exclusion step were pooled and applied to a hydrophobic Phenyl Sepharose CL-4B column at room temperature, which was operated at 4°C. Under these conditions the PAF-AH activity was completely retained on the column. Most of the contaminating proteins were either not bound to the resin or eluted stepwise with a buffer containing CHAPS, at a concentration of 3.3–8 mM. The enzyme was eluted as a single sharp peak with 10 mM CHAPS (Fig. 1B), suggesting the extremely hydrophobic nature of the mouse platelet PAF-AH. The subsequent hydroxylapatite batch elution step was performed in the presence of 10 mM CHAPS (Fig. 1C), because in lower CHAPS concentrations, the activity was not eluted from the hydroxylapatite, even at 600 mM potassium phosphate. The active fractions from the batch hydroxylapatite step after ultrafiltration and lyophilization were subjected to a 7.5% polyacrylamide gel electrophoresis under non-denaturing conditions (Native-PAGE), in the presence of CHAPS. Under these conditions PAF-AH activity was recovered as a single sharp peak with an Rf value of 0.12 (Fig. 1D). In this step, a significant

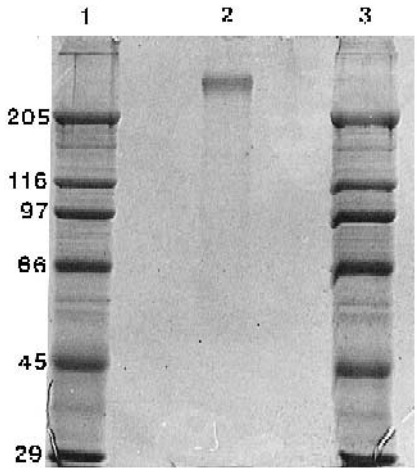

Fig. 2. SDS-PAGE in 7.5% acrylamide, under non-reducing conditions of the purified membrane-associated PAF acetylhydrolase of mouse platelets. Lanes 1 and 3, molecular weight markers (kDa). Lane 2, 3.5 μg of the purified product. Proteins were stained with silver nitrate.

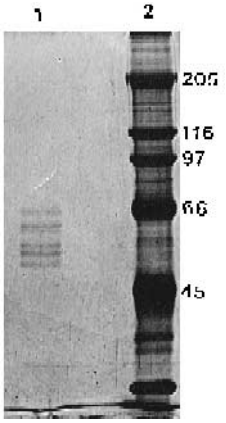

Fig. 3. SDS-PAGE in 7.5% acrylamide, under reducing conditions of the purified PAF acetylhydrolase from platelet membranes. Lane 1, 1 μg of the purified product. Lane 2, molecular weight markers (kDa). Proteins were stained with silver nitrate.

loss of the activity was observed. Subsequent SDS-gel electrophoresis (7.5% acrylamide and non-reducing conditions) of the active peak showed a single protein band with an estimated molecular weight of 270 kDa (Fig. 2). Under reducing conditions as described in Section 2, this protein band was resolved into four bands and one duplet with estimated molecular weights of 66, 55, 52, 49 and 62 kDa, respectively (Fig. 3). The summary of the purification procedure is shown in Table 3.

3.4. Effect of temperature on PAF-AH

The PAF-AH activity of the final purification product decreased with temperature; it was reduced by 85% after a 10-min preincubation period at 60°C (Fig. 4A). Similar thermolability was observed for

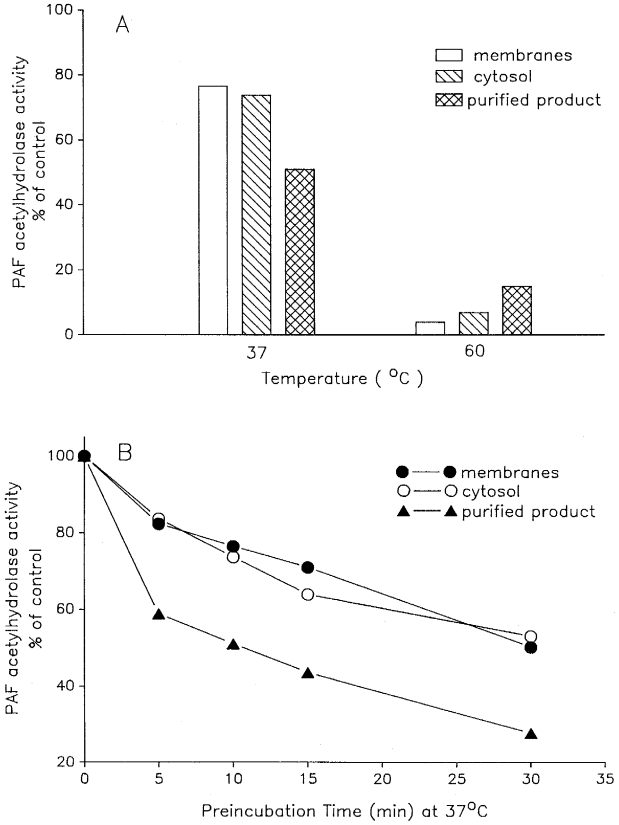

Fig. 4. Inactivation of PAF acetylhydrolase as a function of temperature (A) or preincubation time at 37°C (B). A preparation of the final purification product (0.2 μg of protein) was preincubated for 10 min at the indicated temperature (A) or for the indicated time at 37°C (B). Then [^{3}H]acetyl-PAF (10 μM) was added and PAF acetylhydrolase was assayed as described in Section 2. The inactivation of PAF acetylhydrolase activity in crude membrane (5 μg of protein) and cytosolic (10 μg of protein) preparations of mouse platelets were also included for comparison. Control activity in these experiments was 342, 52 and 44 nmol/h/mg for the purified product, the membranes and the cytosol respectively.

Table 3
Purification of PAF acetylhydrolase from mouse platelet membranes

Step	Protein (mg)	Total activity		Specific activity (nmol/h/mg)	Purification
		nmol/h	%		
Lysate	19.10	1112		58.2	
104 000 × *g* pellet	7.30	638		87.4	
Solubilized membranes	6.13	460	100	75.0	1
Superose 6B	3.32	611	133.0	184	2.5
Phenyl Sepharose CL-4B	0.43	327	71.1	761	10.1
Hydroxylapatite	0.25	250	60.9	1000	13.3
Ultrafiltration	0.091	144	31.3	1586	21.1
Native-PAGE	0.028	16.5	3.6	588	

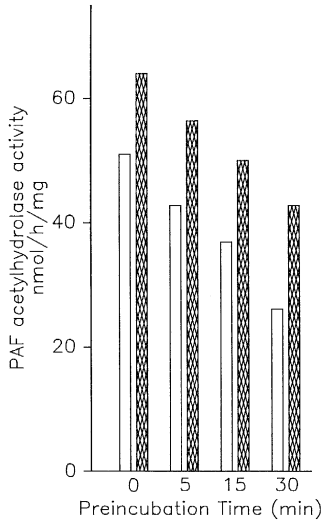

Fig. 5. Effect of DTE on PAF acetylhydrolase activity as a function of time. A platelet membrane preparation containing 5 μg of protein was preincubated with 5 mM DTE for the indicated time (0–30 min) at 37°C. Then [^{3}H]acetyl-PAF (10 μM) was added and the PAF acetylhydrolase activity was assayed as described in Section 2. Control activity in open bars and activity after treatment with DTE in hatched bars.

the crude membrane and cytosolic preparations of mouse platelets (Fig. 4A). Fig. 4B shows the loss of the activity as a function of preincubation time at 37°C. There was a similar decrease of the activity for the membrane and the cytosolic fraction of mouse platelets and a 50% loss of the enzymatic activity was observed when the preincubation was carried out for 30 min. The purified product was significantly more susceptible to temperature inactivation since 50% of the enzymatic activity was already lost after preincubation for 5 min at 37°C and only 27% of the activity was retained after 30 min at this temperature.

The presence of reducing agents, such as DTE (5 mM), in the crude preparation of platelet membranes, in spite of the induced enhancement of the enzymatic activity, was not able to protect the enzyme from the temperature inactivation (Fig. 5). The rate of the enzymatic activity loss was the same, in the presence or absence of DTE, suggesting that temperature affects the structure of the enzyme as a whole, rather than groups, essential for the enzymatic activity.

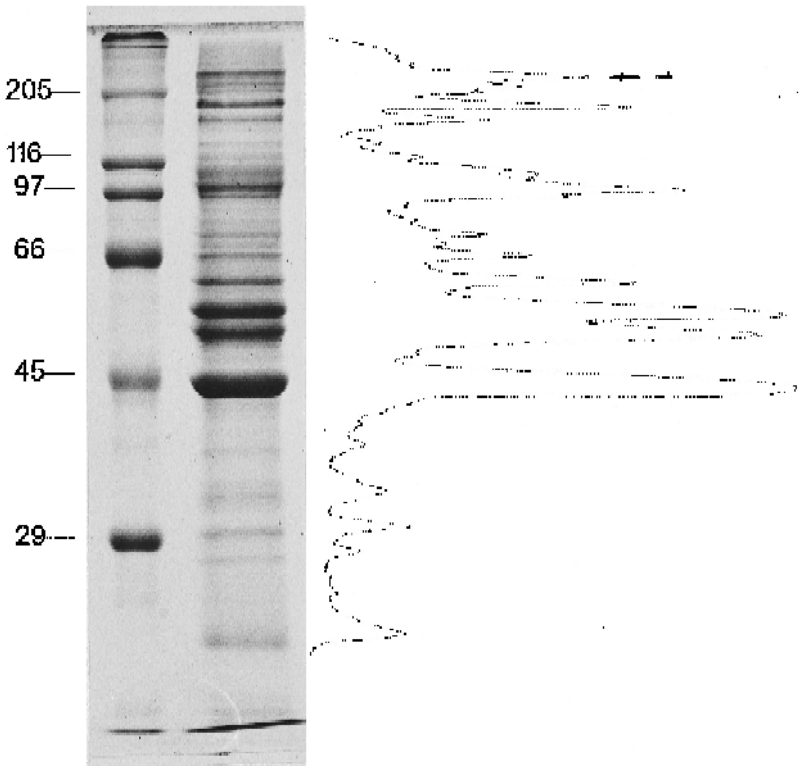

Fig. 6. Densitogram of the solubilized mouse platelet membranes. A preparation of solubilized platelet total membrane fraction, containing 65 μg of protein, was subjected to SDS-PAGE (10% acrylamide, under non-reducing conditions), as described in Section 2. Peak 1 corresponds to the band having a molecular weight of 270 kDa.

3.5. Densitometric analysis of platelet membrane proteins

Fig. 6 shows the profile of a solubilized membrane preparation on SDS-PAGE, performed in 10% acrylamide under non-reducing conditions and its densitometric analysis. The intensity of the band which corresponds to the molecular weight of 270 kDa, the estimated molecular weight of the PAF-AH, was found to constitute approx. 2.3% of the total Coomassie-stained protein.

4. Discussion

In this work we demonstrated that mouse platelets contain PAF-AH activity, which is present to the extent of 57% in the total membrane fraction and 43% in the cytosol. This finding is in contrast with other blood cells and tissues of the various species studied to date, in which PAF-AH activity has been found mainly in the cytosol [10,11]. Nevertheless, in the protozoan *Tetrahymena pyriformis* most of the activity was detected in the microsomal fraction [28] and furthermore, in Ehrlich ascites cells, increased activity was found in the microsomes [29]. Furthermore a particulate fraction-associated PAF-AH activity has been reported in HL-60 cells and an increase in its relative distribution to this fraction during differentiation has been observed [30]. As for the platelets of other species, the presence of PAF-AH activity in their membrane fraction has not to date been investigated.

The very low apparent K_m value of 0.8 μM for both the membrane-associated and the cytosolic enzymatic activity (Table 1), suggests the presence of the same enzyme with a very high affinity for PAF in both fractions. The similarity of response of the two enzyme preparations towards temperature (Fig. 4) and various agents (Table 2), is consistent with such a suggestion. The small differences observed for certain compounds, e.g., Ca^{2+} and PC, might be attributed to the different environment of the enzyme in these two fractions.

Mouse platelet PAF-AH is an enzyme totally different from the typical cytosolic or membrane-bound PLA_2, as is shown from: (a) its inhibition rather than its activation by Ca^{2+} and its slight stimulation by EDTA, (b) its failure to hydrolyze phosphatidylcholine and, (c) its sensitivity at 37°C [31]. We cannot exclude the possibility that the inhibition by Ca^{2+} and the stimulation by EDTA may be due to Ca^{2+}-dependent proteases present in platelets [32].

The enzyme does not seem to be a lysophospholipase. This is supported by the lower K_m value towards PAF, compared to the lysophospholipase II isolated from bovine liver [33]. Moreover, the response to PMSF, NEM and metal ions differentiates this enzyme from the lysophospholipases of the type isolated from mouse macrophages [34]. It is worth mentioning that in the case of human and rat platelets, PAF-AH and lysophospholipase activities harbor different proteins, as has been reported by Aarsman et al. [33].

The effect of the sulfhydryl agents DTNB, NEM and DTE suggests that the platelet enzyme contains essential sulfhydryl groups and requires the addition of reducing agents for maximal activity. The sensitivity to PMSF suggests the presence of serine residues at the active site. The inhibitory effect of Triton X-100 when the PAF concentration was 10 μM, well above its CMC (2.5–3 μM) [35], could be due to the surface dilution kinetic effect, described by Stafforini et al. [12,13]. Anionic detergents such as DOC-Na and SDS, at concentrations much lower than their CMC, apparently denature the enzyme, possibly by affecting its tertiary and quaternary structure.

We purified, to near homogeneity, the PAF-AH from mouse platelet membranes. The final purification product gave, on SDS-PAGE, one band with an apparent molecular weight of 270 kDa (Fig. 2). This band, under reducing conditions, was resolved into four well separated bands and one duplet with molecular weights of 66, 55, 52, 49 and 62, kDa, respectively (Fig. 3). At this point though, we do not know which of these polypeptides functions as the catalytic site.

The mouse platelet PAF-AH is characterized by an extreme sensitivity at 37°C (Fig. 4B), since a 73% loss of its activity was observed after a 30-min preincubation period at 37°C. In addition, the mouse platelet enzyme is more susceptible to inactivation at 60°C (Fig. 4A) from the erythrocyte enzyme [13].

The instability of mouse platelet PAF-AH, in the crude as well as in the purified preparation, at 37°C was a major problem in the estimation of its actual

specific activity in every step of the purification procedure. Thus the extent of purification (Table 3) based on specific activity can only serve as an indicative marker of this procedure. The final purification fold (21) was calculated from the step prior to native-PAGE, because during this step a significant loss of the activity was observed, as it has been reported by Stafforini et al. [13] for the PAF-AH of human erythrocytes. Moreover Aarsman et al. [33] reported that the recovery of the PAF-AH activity residing in the bovine liver lysophospholipase II was very low, less than 10%, following nondenaturing gel electrophoresis. Our final product (28 μg) constitutes 0.5% of the total initial solubilized protein (6.1 mg) (Table 3). This fact suggests that the enzyme is abundant in mouse platelet membranes. The densitometric analysis of the crude solubilized preparation after SDS-PAGE (Fig. 6) further supports such a suggestion. Therefore, the instability of the enzyme during the purification procedure and the fact that the purification was performed using a starting material already rich in the enzyme, resulted in a final preparation with a low specific activity and consequently a low purification fold. However, the homogeneity of the final preparation as to its PAF-AH content, seems to be approx. 100% (Fig. 2).

From the above, we may conclude that mouse platelet PAF-AH is an enzyme different from the PAF-AHs of normal mammalian cells examined so far. This is supported by the following points: (a) the high concentration of the enzyme in the membranes, (b) the very low K_m value, and (c) its thermolability. In addition the 270 kDa mouse platelet PAF-AH is a protein of higher molecular weight and of a more complex structure than those of the PAF-AHs isolated from other cells and tissues [12–15]. However, the PAF-AHs in the platelets of other species have not been investigated to date and as such, molecular weight comparisons cannot be directly made. Moreover, mouse platelet PAF-AH is an enzyme with properties distinct from the mainly HDL-associated plasma enzyme as is shown by their different K_m, response to various agents, thermosensitivity, molecular weight and structure [36]. We may mention, however, that the 62 kDa polypeptide of the platelet PAF-AH has the same molecular weight with one of the mouse plasma enzymes [36]. Despite the above noted differences, mouse platelet enzyme does share some properties with other cellular and tissue PAF-AHs, e.g., the presence of essential sulfhydryl groups and the strong inhibition by serine hydrolase inhibitors, such as PMSF.

The unexpected presence of PAF-AH activity in platelet membranes led us to examine the possibility of its loose association with the membranes under our experimental conditions. Successive treatments of platelet membranes with a high ionic strength buffer (buffer A, containing 0.5 M NaCl), not only failed to extract the PAF-AH from the membranes, but further increased its specific activity by 100%. This observation suggested that the PAF-AH activity was probably firmly associated with the platelet membranes. Given its presence in both cellular compartments, membranes and cytosol, it remains unknown whether there is a mechanism that allows the migration of the enzyme between these two compartments. We also cannot exclude the possibility that mouse platelet PAF-AH is a membrane-bound enzyme which during sonication, the most effective manner of platelet disruption in our hands, is partially solubilized into the cytosol, as is the case with other membrane-bound enzymes [37].

Intact mouse platelets do not hydrolyze exogenously added PAF, suggesting that PAF-AH (or its active site) is not localized at the outer surface of the platelet membrane and it is not released in the medium. Moreover, mouse platelets, when stimulated by thrombin, do not biosynthesize PAF (unpublished data). Thus, the following two questions arise: what is the role of the high levels of PAF-AH in mouse platelets and what is the significance of its presence in both cytosol and membranes? It could be suggested that mouse platelet PAF-AH utilizes as substrates, in addition to PAF, other PAF-like lipids or oxidized phospholipids, as has been reported in the case of several cells and tissues [16,13–15], and which in this case could be localized in different cellular compartments. Another possibility, which may explain the high concentration of the PAF-AH in mouse platelets and its high molecular weight, is that the enzyme constitutes a functional domain of a multifunctional protein or a multi-enzyme complex. The analysis of the PAF-AH on SDS-PAGE under reducing conditions into six polypeptides (Fig. 3) could support such a hypothesis. Thus, the investigation of the cellular compartmentalization of the PAF-

AH, its structure and its cellular substrates, other than PAF, are necessary for the understanding of the role of this enzyme in mouse platelets.

This is the first report of a purified PAF-AH associated with the membranes of a normal mammalian cell. Our work does not address directly the problem of the mouse platelet refractoriness to PAF [18]. Nevertheless the finding that the PAF-AH is found not only in the cytosol but also bound to the membranes, may finally provide the basis for approaching the problem of mouse and rat platelet nonresponsiveness to PAF [18,38].

Acknowledgements

We thank Prof. D. Tsernoglou and Drs. S. Loukas and M. Mercouris for their helpful discussions.

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