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Review

Do membrane-bound enzymes access their substrates from the membrane or aqueous phase: interfacial versus non-interfacial enzymes

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Abstract

For membrane-bound enzymes that act on substrates that partition between the membrane and aqueous phases, it is possible to imagine two fundamentally different mechanisms. *Interfacial* enzymes must access their substrate from the membrane phase, in other words substrate in the membrane binds directly to the active site of the enzyme at the membrane without mixing with substrate molecules in the aqueous phase. On the other hand, *non-interfacial* enzymes, either bound to membranes or present in the aqueous phase, must access their substrates from the aqueous phase, i.e. substrate in the aqueous phase binds directly to the enzyme without mixing with substrates in the membrane phase. An interfacial mechanism for some enzymes including secreted and cytosolic phospholipase A_2 and phosphoinositide 3′-hydroxykinase was rigorously proven by demonstrating that these enzymes processively hydrolyze many phospholipids without desorbing from the surface of vesicles (scooting mode). The non-interfacial mechanism is more difficult to establish because it cannot be addressed by steady-state kinetics. Using a pre-steady-state method in which the enzymatic velocity is measured during the time it takes for substrate to exchange between vesicles, a non-interfacial mechanism was proven for vesicle-bound plasma platelet activating factor acetylhydrolase. This enzyme prefers more water-soluble phospholipids such as those with *sn*-2 acetyl or oxidatively truncated fatty acyl chains, and this is readily explained by the mandatory access of substrate from the aqueous phase.

Keywords: Secreted phospholipase A_2; Platelet activating factor acetylhydrolase; Interfacial enzymology; Membrane protein; Receptor-ligand interaction

1. Interfacial versus non-interfacial enzymology

For membrane-bound enzymes that act on substrates that exist in both the water and membrane compartments of biological systems, it becomes interesting and challenging to determine if the enzyme must access its substrate from the membrane phase or from the aqueous phase. It is a problem of general interest because the same issue arises with receptors for partially lipid-soluble ligands. This distinction is best illustrated with reference to the scheme in Fig. 1. The scheme shows a water-soluble enzyme that associates with the membrane (interfacial binding). We are concerned here with the properties of a peripheral membrane-bound enzyme, and our arguments apply equally well to integral membrane proteins that never exist in a water-soluble state.

A peripheral membrane-bound protein in its

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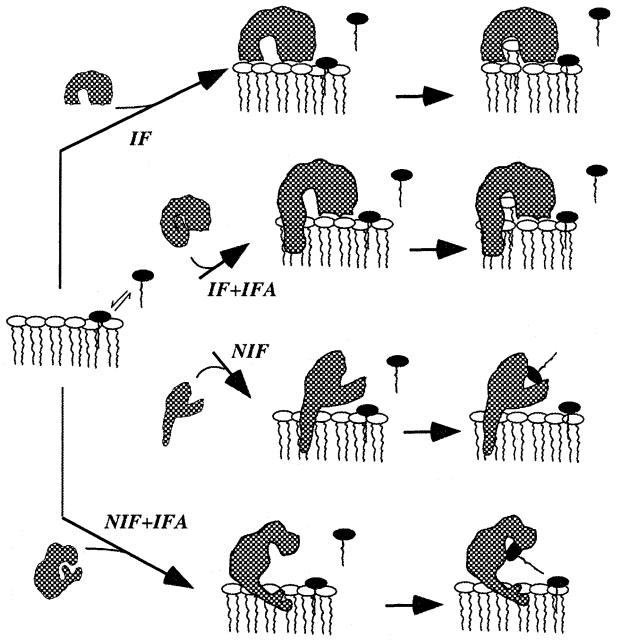


Fig. 1. Enzymes that act on the membrane-water interface or in the aqueous phase. Water-insoluble phospholipid is shown in white, and a substrate that partitions between the membrane and water phases is shown in black as a single chain amphiphile. Interfacial mechanism (IF, top line): the enzyme at the interface acts on substrate at the interface (either white or black substrate, only action on white is shown for simplicity). Only substrate in the interface binds to the catalytic site. Interfacial with interfacial activation mechanism (IF+IFA, second line from top): similar to the IF mechanism except that the enzyme undergoes an allosteric transition upon binding to the interface which increases its catalytic efficiency to act on substrate at the interface. Non-interfacial mechanism (NIF, third line from top): only substrate in the aqueous phase can bind to the catalytic site of the enzyme at the interface. Non-interfacial with interfacial activation mechanism (NIF+IFA, bottom line): similar to the NIF mechanism, but enzyme undergoes an allosteric transition upon binding to the interface which activates it toward the substrate in the aqueous phase.

water-soluble state may or may not be catalytically active on water-soluble substrate in the absence of membranes. However, two possibilities exist for the membrane-bound form of this enzyme as shown in Fig. 1. The *interfacial enzyme* can access its substrate only from the membrane phase. In other words, substrate in the aqueous phase cannot directly bind to the active sites of the enzyme without first passing through the membrane phase, and substrates in the membrane bind directly to the active site of the enzyme without mixing with the substrate molecules in the aqueous phase. In contrast, the *non-interfacial enzyme* at the interface can access its substrate only from the aqueous phase, i.e. the water-soluble substrates can bind directly to the active site of the membrane-bound enzyme without mixing with substrates in the membrane phase.

As also shown in Fig. 1, binding of the enzyme to the membrane may cause an allosteric activation that makes the enzyme a more efficient catalyst (larger k_{cat}/K_m for a particular substrate), and this is termed *interfacial activation*. Thus, we can imagine four types of enzymatic processes: interfacial mechanisms with and without interfacial activation (IF+IFA and IF, respectively) and non-interfacial mechanisms with and without interfacial activation (NIF+IFA and NIF, respectively). For integral membrane proteins, we can make the distinction between IF and NIF mechanisms, but interfacial activation cannot be addressed because the activity of the enzyme in the absence of membrane cannot be measured (a type of interfacial activation whereby specific phospholipids in the membrane activate the integral membrane enzyme is possible). Operationally, it can be said that water-soluble enzymes that never exist in a membrane-bound state operate only by a NIF mechanism.

2. Interfacial enzymes

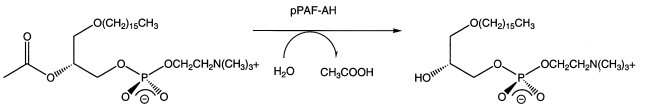
An example of an IF enzyme is secreted phospholipase A_2 ($sPLA_2$). Since $sPLA_2$s work on naturally occurring long-chain phospholipids, i.e. with *sn*-1 and *sn*-2 fatty acyl chains typically of 16 carbons or more, it is reasonable to propose that such enzymes would operate by an IF mechanism. This is because the concentration of substrate in the aqueous phase is vanishingly small, and thus the enzyme should access its substrate only from the membrane phase. This IF behavior is intrinsic in the interfacial enzyme kinetic model put forth by De Haas and coworkers [1] as well as in the dual phospholipid model proposed by Dennis and co-workers [2]. However, the only experimental method so far described that proves that an enzyme operates by an IF mechanism is to establish whether the enzyme undergoes processive interfacial catalysis, also known as catalysis in the *scooting mode* [3,4]. In the scooting mode, $sPLA_2$ catalyzes the hydrolysis of many phospholip-

ids without dissociating from the membrane into the aqueous phase and without exchange of substrates between vesicles. Scooting is the two-dimensional equivalent of one-dimensional processivity, or sliding, of DNA polymerase, which catalyzes the incorporation of several nucleotide triphosphates into a growing daughter DNA strand while remaining bound to the template DNA strand.

Two-dimensional scooting behavior is straightforward to investigate. For example, if there is no exchange of $sPLA_2$ or phospholipid substrates and products between phospholipid vesicles, then addition of vesicles of a radiolabeled phospholipid to vesicles of the same phospholipid but in non-radiolabeled form and containing bound $sPLA_2$ does not lead to radioactive products because enzyme cannot hop to radioactive vesicles and radiolabeled phospholipids cannot transfer to enzyme-containing vesicles. Likewise, radioactive products are produced from vesicles of radioactive phospholipid containing bound $sPLA_2$, and no reduction in rate of radioactive product formation occurs upon addition of vesicles of non-radioactive phospholipids. Clearly, if the enzyme were acting on trace amounts of phospholipid in the aqueous phase, this type of processive behavior would not be possible. To the best of our knowledge, scooting mode behavior has been observed only for the following enzymes: (1) all $sPLA_2$s that have been examined for scooting [3,5,6]; (2) cytosolic (group IV) phospholipase A_2 [7]; (3) phosphoinositide 3′-hydroxykinase [8]; (4) a bacterial lipase with broad specificity [9]; (5) bacterial sphingomyelinase (D. Zakim and M. Jain, unpublished).

3. Establishment of the non-interfacial mechanism requires novel approaches

Proof that an enzyme operates by a NIF mechanism is much more difficult to obtain, and to the best of our knowledge this has been accomplished only for the enzyme human plasma platelet activating factor acetylhydrolase (pPAF-AH) [10]. Platelet activating factor (PAF) is a phosphatidylcholine with an *sn*-1 hexadecyl or octadecyl ether and an *sn*-2 acetyl group. pPAF-AH displays PLA_2 activity since it hydrolyzes the *sn*-2 ester to produce acetate and lyso-PAF. Since lyso-PAF is devoid of the pro-inflammatory activities



of PAF [11], the reaction catalyzed by pPAF-AH is of interest for understanding how PAF-mediated signaling is terminated in vivo [12,13].

pPAF-AH is found in the plasma associated with lipoproteins (LDL and HDL) [12]. It was identified and purified based on its ability to hydrolyze PAF but not phospholipids with two long-chain fatty acyl groups. In fact, activity of this enzyme progressively falls as the length of the substrate *sn*-2 chain increases; activity is close to zero with *sn*-2 chains of approximately eight carbons or longer [12]. Subsequently, it was found that pPAF-AH also works well on phosphatidylcholines with oxidatively truncated *sn*-2 fatty acyl chains, i.e. those bearing a polar COOH or CHO group at the ω-end of their *sn*-2 fatty acyl chains, suggesting that this enzyme plays a role in the removal of oxidized phospholipids [12].

Since PAF and oxidatively truncated phosphatidylcholines are much more soluble in water than phospholipids with two long-chain fatty acyl groups, it seems possible that pPAF-AH operates by a NIF mechanism, i.e. membrane-bound pPAF-AH can only access substrates from the aqueous phase as illustrated in Fig. 1. The other possibility is that pPAF-AH is an IF enzyme with an active site architecture that only accommodates phospholipids with short *sn*-2 fatty acyl chains. The difficulty in determining whether pPAF-AH is an IF or a NIF enzyme lies in the fact that all types of steady-state kinetic experiments cannot distinguish between these two mechanisms. This is because the concentrations of PAF substrate in the membrane phase and in the aqueous phase are linked by a constant, the partition equilibrium constant for the transfer of substrate between the two phases (K').

$$K' = [\mathrm{PAF}]_w / X_{\mathrm{PAF}}$$

Here $[\mathrm{PAF}]_w$ is the molar concentration of PAF in the water phase, and X_{PAF} is the mole fraction of PAF in the membrane. Thus, if we assume a NIF mechanism for pPAF-AH and write the standard

Michaelis-Menten equation in terms of the concentration of substrate in the aqueous phase:

$$v_i = V_{max}\,[PAF]_w/([PAF]_w + K_m) =$$

$$V_{max}\,K'\,X_{PAF}/(K'\,X_{PAF} + K_m)$$

we realize immediately that we can write an equivalent equation in terms of the membrane concentration of substrate. Since the values of the steady-state constants V_{max} and K' are not known a priori, strictly speaking we have no way to know which of the two Michaelis-Menten equations shown above apply. In other words, suppose we measure the velocity of membrane-bound pPAF-AH as a function of the reaction mixture buffer volume in the presence of a fixed amount of PAF substrate and membrane lipid. As we increase the buffer volume, the concentration of PAF in the aqueous phase drops and the velocity will drop in a hyperbolic fashion according to the Michaelis-Menten equation. However, the concentration of PAF in the membrane will also drop as will the enzymatic velocity in a hyperbolic manner. This is true in the case that substrate exchanges rapidly between the membrane and aqueous phases.

The inability of steady-state kinetics to resolve IF versus NIF mechanisms can also be illustrated by describing experiments with pPAF-AH carried out with the PAF analog with a decyl *sn*-1 chain (C_{10}-PAF) [10]. For these studies, pPAF-AH bound to vesicles of dimyristoylphosphatidylcholine (DMPC) was used to mimic the enzyme bound to the phosphatidylcholine-rich surface of lipoproteins. By monitoring fluorescence resonance energy transfer between enzymic tryptophans and a dansylated phospholipid present in trace amounts in DMPC vesicles, it was shown that pPAF-AH binds essentially irreversibly to these vesicles (no interfacial dissociation seen in 30 min) [10]. The enzymatic velocity for C_{10}-PAF hydrolysis was measured with a fixed amount of substrate in the presence of an increasing concentration of DMPC vesicles. At low vesicle concentrations (20 μM) most of the C_{10}-PAF is in the aqueous phase and the membrane concentration of substrate is low. If the vesicle concentration is doubled, the concentration of C_{10}-PAF in the membrane phase will remain constant since both the moles of C_{10}-PAF in the membrane and the number of vesicles will double. The aqueous phase concentration of substrate will remain approximately constant since most of the C_{10}-PAF is in the aqueous phase. Thus for both the IF and NIF mechanisms, the reaction velocity will not change much. With DMPC concentrations above about 2 mM, most of the C_{10}-PAF is in the vesicles. If the DMPC concentration is now doubled, the concentration of substrate in both phases will drop by a factor of 2 and the enzymatic rate will drop regardless of whether the IF or NIF mechanisms apply. These phenomena have been observed for pPAF-AH [10].

The rate of hydrolysis of *p*-nitrophenyl acetate by pPAF-AH bound to 50 μM DMPC vesicles is slightly higher (1.3-fold) than the rate measured in the absence of vesicles [10]. With 50 μM DMPC present, most of the *p*-nitrophenyl acetate is in the aqueous phase, and thus the comparison of these two rates allows the catalytic efficiency of the membrane-bound and aqueous phase forms of pPAF-AH to be compared in a meaningful way, i.e. in the presence of the same aqueous phase concentration of substrate in both cases. Thus, it can be said that pPAF-AH does not undergo significant interfacial activation when bound to DMPC vesicles. These results rule out the IF+IFA and NIF+IFA mechanisms (Fig. 1) for pPAF-AH.

4. Hints of a non-interfacial mechanism for pPAF-AH

PAF-AH also catalyzes the hydrolysis of PAF and PAF analogs in the absence of a lipid-water interface. The critical micelle concentration (CMC) of C_{14}-PAF has been reported to be in the range 11–46 μM using different techniques [10,14]. Fig. 2 shows the initial rate of hydrolysis of C_{14}-PAF by pPAF-AH in the absence of vesicles and as a function of the substrate concentration. Below the CMC, the rate increases linearly with the concentration of C_{14}-PAF indicating that the K_m value for this substrate is well above CMC (i.e. no evidence for saturation kinetics). Fig. 2 also shows that the rate is independent of the C_{14}-PAF concentration above 20 μM (close to the CMC). Note that the transition is abrupt; the curve is not described well by a hyperbola. This result is consistent with a NIF mechanism for pPAF-AH. If enzyme operates only on substrate

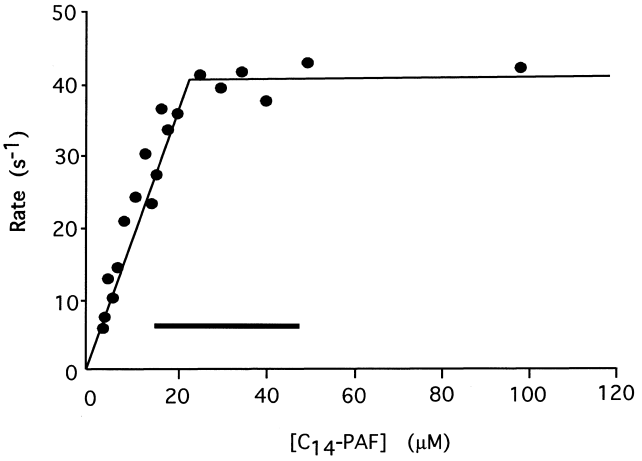


Fig. 2. Initial velocity (pH-stat) for the hydrolysis of C_{14}-PAF by pPAF-AH as a function of substrate concentration in the absence of phospholipid vesicles (based on Fig. 3 of [10], reprinted with permission from the American Chemical Society). The experimental range of values of CMC for C_{14}-PAF is indicated by the horizontal bar.

in the aqueous phase, the horizontal part of the curve in Fig. 2 is due to the fact that the concentration of aqueous phase substrate does not increase beyond the CMC as more and more lipid is added, and thus the rate does not change.

The possibility of an interfacial mechanism for pPAF-AH cannot be conclusively abandoned based on the data in Fig. 2. One could argue that more and more enzyme is drawn into protein-lipid microaggregates as the concentration of C_{14}-PAF is increased until all of the enzyme is bound to lipid micelles (linear portion of the curves). At that point, addition of more micelles will not increase the rate further since it will not change the surface concentration of substrate that micellar-bound enzyme encounters (mole fraction 1). Formation of protein-lipid microaggregates occurs with $sPLA_2$s in the presence of short-chain phospholipids present at concentrations below their CMCs [1,15,16]. A substrate concentration rate profile similar to that shown in Fig. 2 has been observed for a lysophospholipase isolated from a macrophage cell line [17] suggesting, but not proving, that this enzyme operates by a NIF mechanism.

Surprisingly, it was found that increasing the length of the *sn*-1 chain of PAF from 16 to 24 carbons causes a significant reduction (100-fold) in the rate of hydrolysis by pPAF-AH. These studies were carried out in the presence of DMPC vesicles and most of the C_{16}-PAF and C_{24}-PAF are partitioned into the membrane phase. Thus, pPAF-AH appears to sense the length of the *sn*-2 and *sn*-1 fatty acyl chains of phosphatidylcholines. It seems unlikely that the active site architecture of pPAF-AH is designed to discriminate against both long chains on the glycerol backbone. The alternate explanation is that the enzyme works by a NIF mechanism, and thus the rate for C_{24}-PAF hydrolysis is reduced by the fact that the concentration of this substrate in the aqueous phase is lower than that of C_{16}-PAF.

With $sPLA_2$, the rate of C_{24}-PAF hydrolysis is only 1.2-fold lower than for C_{16}-PAF. This is not surprising since $sPLA_2$ is an interfacial enzyme. Since both C_{16}-PAF and C_{24}-PAF are mostly in the membrane, the surface concentrations of these two substrates are similar. Furthermore, the length of the active site of $sPLA_2$ is equivalent to an alkyl chain of about eight carbons. Thus the interactions of C_{16}-PAF and C_{24}-PAF with the active site of $sPLA_2$ on the membrane should be virtually identical, the major difference being the number of carbons that protrude from the protein and left in the membrane phase.

5. Proof that pPAF-AH operates by a non-interfacial mechanism

The key to determining whether pPAF-AH operates by a NIF mechanism is to carry out pre-steady-state experiments in which the enzymatic velocity is measured during the time it takes for the substrate to desorb from vesicles. Kinetic studies were carried out with the fluorescent phosphatidylcholine analogs C_6-NBD-PC and C_{12}-NBD-PC that bear an NBD fluorophore at the ω-end of the 6- or 12-carbon, respectively, *sn*-2 fatty acyl chain. These phospholipid analogs are hydrolyzed by pPAF-AH, albeit slowly, and the rate of intervesicle exchange of these substrates can be accurately monitored by fluorescence methods [18]. The half-time for intervesicle exchange of C_6-NBD-PC and C_{12}-NBD-PC is 0.6 and 60 min, respectively [10]. Studies with the more slowly exchanging substrate C_{12}-NBD-PC were carried out in the presence of dimyristoylphosphatidylmethanol (DMPM) vesicles. Like DMPC, DMPM supports essentially irreversible interfacial binding of pPAF-

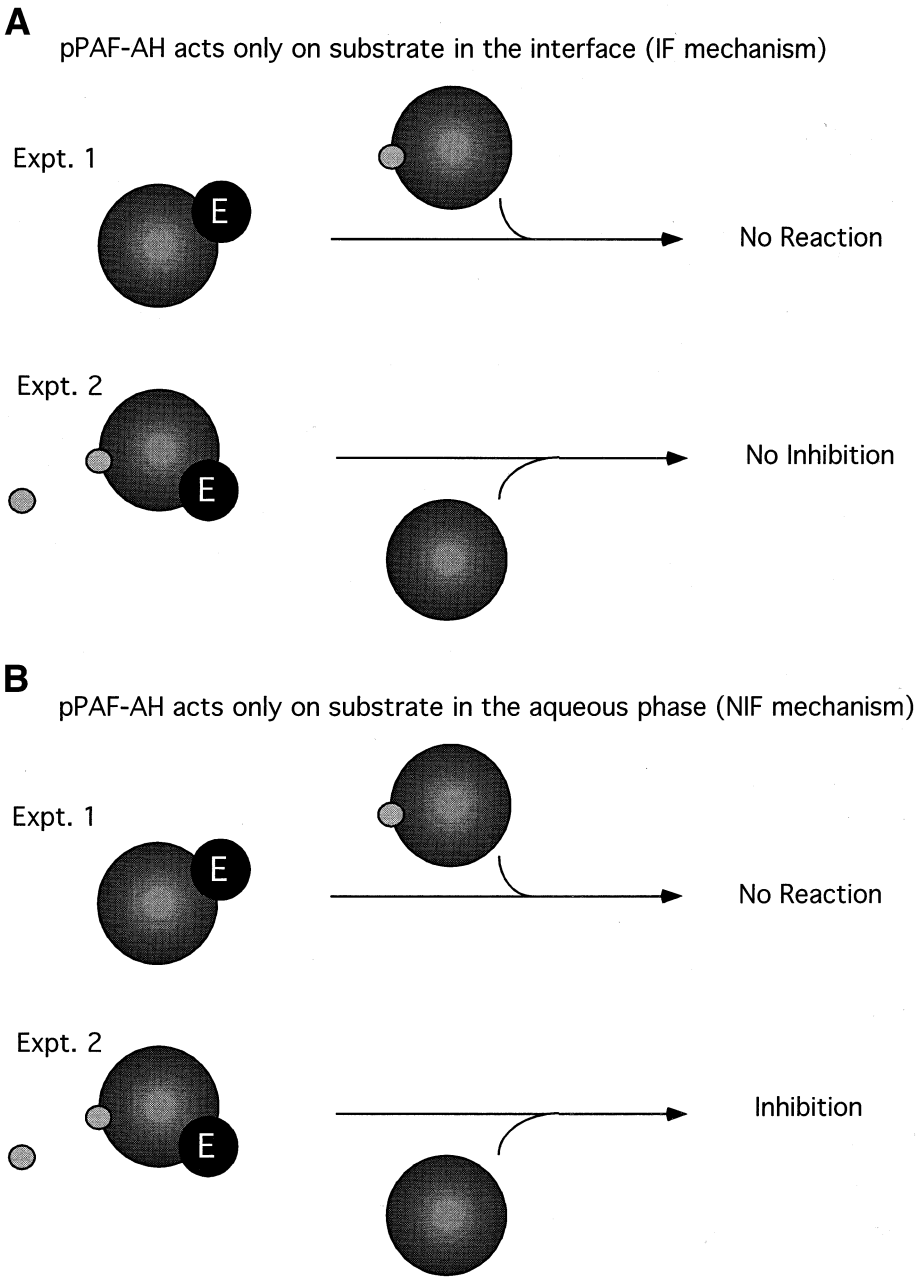


Fig. 3. Schematic showing the expected pre-steady-state kinetic results for the IF mechanism (A) and the NIF mechanism (B). Phospholipid vesicles are shown as large gray spheres, substrates are shown as small light gray spheres, and enzymes are shown as black spheres labeled E.

AH. Enzymatic hydrolysis of C_{12}-NBD-PC can be followed either fluorimetrically or with a pH-stat.

The experimental approach schematically illustrated in Fig. 3 allows the NIF and IF mechanisms to be distinguished. Fig. 3A shows the system for the IF mechanism. In experiment 1, pPAF-AH is prebound to DMPM vesicles, and DMPM vesicles containing a small amount of C_{12}-NBD-PC are added last from a concentrated stock solution. Immediately after addition of the second vesicles, the portion of C_{12}-NBD-PC in the stock solution that is in the aqueous phase is immediately diluted well below the K_m for pPAF-AH. Since it takes tens of minutes for C_{12}-NBD-PC in the vesicle to transfer to enzyme-

containing vesicles (enzyme cannot transfer to C_{12}-NBD-PC-containing vesicles), there will be no pPAF-AH-catalyzed C_{12}-NBD-PC hydrolysis in the first few minutes. As shown in Fig. 3B (experiment 1) for the NIF mechanism, no enzymatic reaction will occur in the first few minutes because the concentration of C_{12}-NBD-PC in the aqueous phase is very low just after adding substrate-containing vesicles. Thus, experiment 1 does not distinguish the IF and NIF mechanisms. Experiment 2 starts with enzyme prebound to DMPM vesicles containing C_{12}-NBD-PC. For the IF mechanism, pPAF-AH is hydrolyzing substrate in the vesicle. After addition of DMPM vesicles without C_{12}-NBD-PC there is no immediate decrease in the enzymatic reaction velocity because it takes tens of minutes for the substrate in enzyme-containing vesicles to transfer to vesicles lacking enzyme. On the other hand, if the NIF mechanism applies (Fig. 3B), vesicle-bound enzyme is working on the small amount of C_{12}-NBD-PC that is in the aqueous phase. Addition of DMPM vesicles will lead to a rapid drop in the aqueous concentration of substrate as it absorbs into the freshly added vesicles (the association rate constant for absorption of C_{12}-NBD-PC by vesicles is of the order of 10^8 M^{-1} s^{-1}, thus the half-time for uptake of C_{12}-NBD-PC by 2×10^{-8} M vesicles (20 000 lipids per vesicle) is approx. 1 s). Experiment 2 is the diagnostic test for the NIF versus IF mechanisms, and experiments with pPAF-AH show inhibition in experiment 2 [10].

These order of addition effects (experiment 1 versus 2 of Fig. 3) should vanish if substrate undergoes rapid intervesicle exchange, regardless of whether the NIF or IF mechanisms apply. Indeed, the rate of hydrolysis of C_6-NBD-PC by pPAF-AH in the presence of DMPM vesicles is invariant to the order of addition of components [10]. These pre-steady-state experiments outlined in Fig. 3 provide proof that pPAF-AH operates by a NIF mechanism.

6. Implications of the non-interfacial mechanism

The NIF mechanism for pPAF-AH readily accounts for the unusual substrate specificity of this enzyme. It is difficult to imagine an active site architecture that prefers phosphatidylcholine with an *sn*-2 acetyl group over one with say an *sn*-2 pentanoyl chain, and yet addition of a COOH or CHO group to the ω-end of the pentanoyl chain allows the phospholipid to become a good substrate. Also the active site of pPAF-AH would also have to discriminate against long *sn*-1 chains (recall the substrate specificity data presented above). Clearly, good substrates for pPAF-AH have the common feature of better water solubility than phospholipids with two long-chain fatty acyl groups. The NIF mechanism in which pPAF-AH can only access its substrate from the aqueous phase nicely explains the observed substrate specificity.

These results also suggest that pPAF-AH may work as an esterase on a structurally diverse set of esters other than phosphatidylcholines. To begin to explore this, we have found that pPAF-AH is tolerant to a variety of phospholipid polar head groups other than the phosphorylcholine of PAF; the modest selectivity is accounted for by differences in the aqueous-to-vesicle partition constants, which modulates the concentration of substrate in the aqueous phase [10]. It seems less likely that an interfacial enzyme will have broad specificity toward a variety of esters. If the enzyme is able to work on phospholipids with two long-chain fatty acids, it must have special features including an interfacial binding surface to allow it to bind to the membrane interface. In this case, it seems unlikely that the physiological substrates for such an enzyme would be a partially water-soluble ester. On the other hand, the identification of the true physiological substrates for a NIF enzyme is more challenging. This is true not only for pPAF-AH but also for the type of lysophospholipases that hydrolyze partially water-soluble lysophospholipids but not two-chain phospholipids. In this context, it may be noted that a previously identified mammalian lysophospholipase may actually be a thioesterase that removes palmitoyl groups from the α-subunits of heterotrimeric G proteins [19].

The NIF mechanism for membrane-bound pPAF-AH has other implications. For example, it is of interest to determine if the signaling receptor for PAF operates by a NIF or IF mechanism. One possibility is that this integral membrane protein can only bind PAF from the aqueous phase. In this possible scenario, enzymatic exchange of the *sn*-2 long-chain fatty acid of phosphatidylcholine with an acetyl group allows the lipid to enter the aqueous phase

and thus reach the ligand binding site of its receptor. A similar possibility can be considered for the lysophosphatidic acid receptor. Pre-steady-state kinetic experiments can be envisioned to test these hypotheses.

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