Tryptophan-Containing Mutant of Human (Group IIa) Secreted Phospholipase A_2 Has a Dramatically Increased Ability To Hydrolyze Phosphatidylcholine Vesicles and Cell Membranes†

Sharon F. Baker, Roohaida Othman, and David C. Wilton*

Division of Biochemistry and Molecular Biology, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, United Kingdom

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ABSTRACT: Human nonpancreatic (group IIa) secreted phospholipase A_2 (human $sPLA_2$) is associated with a number of inflammatory disorders in which the extracellular concentrations of this enzyme can become highly elevated. It is probable that the enzyme normally acts as an acute-phase protein whose function is to facilitate the removal of infectious organisms or damaged host cells as part of the normal inflammatory response. The enzyme shows negligible activity with phosphatidylcholine (PC) vesicles and cell membranes, presumably reflecting the enzyme's lack of ability to bind productively to such condensed neutral interfaces. Mammalian pancreatic enzymes show modest activity with such interfaces and contain a unique tryptophan at position 3, which is part of the presumptive interfacial binding surface of these enzymes. Human $sPLA_2$ does not contain tryptophan. The amphiphilic indole side chain of tryptophan is noted for its ability to penetrate the lipid interface of membranes, and tryptophan residues appear to be associated with the ability of lipases and phospholipases A_2 to bind to and hydrolyze such interfaces. We have investigated in detail the properties of a V3W mutant of human $sPLA_2$, which has a unique tryptophan on the interfacial binding surface of this enzyme. Although this enzyme shows a modest (∼50%) reduction in activity when anionic substrates are used under standard assay conditions, the activity of the enzyme on phosphatidylcholine vesicles and cell membranes is dramatically increased compared with human $sPLA_2$. This is particularly the case with small unilamellar vesicles of PC, where activity is enhanced over 250-fold compared to the almost zero activity expressed by human $sPLA_2$. This enhanced activity is best explained by increased interfacial binding and activation of the V3W mutant and is not due to enhanced active-site binding and hydrolysis. The results highlight the important role that tryptophan residues can play in interfacial binding, particularly to condensed zwitterionic interfaces. The interfacial characteristics of the mutant human enzyme now resemble more closely the mammalian pancreatic enzymes that already have a tryptophan at position 3.

Nonpancreatic secreted (Group IIa) phospholipases A_2 ($sPLA_2$)[1] are mammalian enzymes that are part of a larger group of structurally related 14 kDa enzymes that hydrolyze the fatty acid ester in the 2-position of phospholipids (for recent reviews, see (refs *1*−*4*)). These group IIa mammalian enzymes are associated with the inflammatory response of tissues as a result of infection or trauma (*5*, *6*). Although these $sPLA_2$s are released by a variety of cells in response to a wide range of inflammatory stimuli, their role in the inflammatory process remains obscure. In the case of human $sPLA_2$, elevated extracellular levels of this enzyme are associated with many inflammatory disorders while blood levels are most acutely raised in association with septic shock (*5*, *6*). However, a direct molecular connection between this enzyme and these disease processes has not been established, and overexpression of this enzyme in transgenic mice failed to produce any significant inflammatory disorder (*7*, *8*). It is probable that this enzyme may be part of the body response to inflammation, possibly as an acute-phase protein (*9*), whose function is to help remove infectious organisms and damaged tissue (reviewed in ref 10).

A characteristic of human $sPLA_2$ is its almost zero activity on neutral PC vesicles, cell membranes (*11*−*14*), and lipoprotein emulsion particles (*15*), a property that would normally prevent high extracellular levels of this enzyme degrading such structures within the body. This property must reflect the inability of the enzyme to bind productively to such condensed zwitterionic interfaces and can be contrasted with the highly destructive role of the snake venom enzymes. For example, the enzyme from the venom of the cobra *Naja naja*, which is structurally very similar to human $sPLA_2$, is able to readily hydrolyze cell membranes (*14*, *16*).

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* Corresponding author: Phone (01703) 594308; FAX (01703) 594459.

[1] Abbreviations: DAUDA, 11-(dansylamino)undecanoic acid; FABP, fatty acid binding protein; PC, phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; PG, phosphatidylglycerol; DOPG, dioleoylphosphatidylglycerol; DOPM, dioleoylphosphatidylmethanol; DTPM, ditetradecylphosphatidylmethanol; human $sPLA_2$, human (group IIa) nonpancreatic secreted phospholipase A_2; MLV, multilamellar vesicle; SUV, small unilamellar vesicle; HBSS, Hanks' balanced salts solution.

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It should be noted that human $sPLA_2$ does not contain tryptophan, while early work on sea snake venom $sPLA_2$ recorded the reduced ability of isoforms that did not contain tryptophan to hydrolyze PC substrates (*17*). Subsequently, it should be emphasized that many of the venom $sPLA_2$s (including that from *N. naja)* have tryptophan residues strategically located on the presumptive interfacial binding surface that includes the N-terminal region of the protein. This has been highlighted in the case of the $sPLA_2$ from *N. naja* and *Crotalus atrox*, where tryptophans at positions 20 and 31, respectively, may be responsible for this role. Moreover, the insertion of a tryptophan into an equivalent position in bovine pancreatic $sPLA_2$ produced a mutant (L20W) with a significant increase in activity on densely packed zwitterionic interfaces (*18*). Tryptophan residues can facilitate membrane penetration as a result of insertion of the planar amphiphilic indole side chain into the lipid interface (*19*).

A characteristic of mammalian pancreatic $sPLA_2$s, not present in the human $sPLA_2$, is the presence of the unique interfacial tryptophan at position 3. This tryptophan has found considerable use as a fluorescent probe for interfacial binding. The fluorescence enhancement and a blue shift in the wavelength of maximum fluorescence emission has been interpreted as the result of desolvation on interfacial binding (*20*). A role of this tryptophan in interfacial binding would therefore be anticipated, and structural evidence to support this has come from mutagenesis studies of the bovine pancreatic enzyme (*21*). To utilize the reporting property of such a tryptophan, we have previously described the preparation and partial characterization of the binding properties of the V3W mutant of recombinant human $sPLA_2$ (*22*).

In this paper, as a part of a comprehensive study of the structural and functional properties of this recombinant human $sPLA_2$ (*14*, *22*−*24*) we have examined in detail the catalytic properties of the V3W mutant. We have established a dramatic enhancement in activity compared to human $sPLA_2$ when SUVs of PC and cell membranes are used as substrates. This enhancement of hydrolysis, due to enhanced interfacial binding, is the most dramatic for any $sPLA_2$ mutant so far described, reflecting the extremely low activity of the wild-type $sPLA_2$ on these substrates. This example provides a clear illustration of the important role played by the amphiphilic aromatic side chain of tryptophan in membrane binding and hence catalysis of interfacial enzymes that act on condensed zwitterionic interfaces.

MATERIALS AND METHODS

Reagents. Chemicals were obtained from BDH (Poole, U.K.) and Sigma (Poole, U.K.). Cell culture reagents were obtained from Gibco−BRL (Paisley, U.K.). DAUDA was obtained from Molecular Probes (Eugene, OR). Porcine pancreatic $sPLA_2$ was obtained from Boehringer Mannheim (Lewes, U.K.). Recombinant FABP was purified as described previously (*25*). Radiochemicals were from Amersham International (U.K.). DTPM was a gift from Professor M. H. Gelb, Seattle, WA.

Human $sPLA_2$. Recombinant human $sPLA_2$ and the V3W mutant were expressed from synthetic genes and purified as described previously (*22*). This recombinant protein and all derived mutants contain an alanine in place of the N-terminal asparagine to allow the *Escherichia coli* aminopeptidase to completely remove the initiator methionine during expression. The properties of this recombinant protein are essentially identical to normal wild-type enzyme (*22*, *23*). Final purity was confirmed by SDS−PAGE and by reverse-phase HPLC, using a method adapted from ref *26*. A 4.6 × 150 mm nucleosil NC33-5C18 column was equilibrated at room temperature with 0.1% (v/v) trifluoracetic acid (TFA) in water and developed at 0.75 mL/min with a linear gradient of acetonitrile (0−60%) in 0.1% TFA. A single peak of active enzyme eluted at 37% acetonitrile.

Circular Dichroism. Circular dichroism spectra of proteins were measured with a Jasco J-720 spectropolarimeter. Enzyme concentrations were 20 μM in 10 mM phosphate buffer, pH 7.4, at 25 °C. Each spectrum was obtained at wavelengths between 195 and 300 nm from 10 scans.

Preparation of Phospholipid Vesicles. Multilamellar vesicles (MLVs) were prepared as follows. One hundred microliters of a 10 mg/mL stock solution of phospholipid in methanol was placed in a glass vial and dried under a stream of nitrogen. The lipid film was resuspended in 1 mL of the appropriate buffer by vortexing for 10 min. In vesicle binding assays, MLVs were pelleted by centrifugation at 400000*g* for 10 min in a Beckman TLX ultracentrifuge. Sonicated phospholipid vesicles were prepared by probe sonication of the MLV preparation using a Heat Systems XL-2020 sonicator. For the measurement of single phospholipid species, SUVs were prepared by the solvent injection method (*14*). Phospholipid substrates used (DOPC, DOPG, and DOPM) were in the form of fluid bilayers.

Preparation of Cells, Rat Liver Plasma Membranes, and Microsomes. RAW 264.7 cells were grown and assayed as described elsewhere (*14*). Plasma membranes were isolated from female Wistar rats (200−250 g) by the method of ref *27*. Microsomal membranes were also prepared from the livers of Wistar rats after homogenization in 10× volume of SET buffer (250 mM sucrose, 1 mM EDTA, and 20 mM Tris·HCl, pH 7.4) following 10 passes of a Dounce homogenizer. The microsomal fraction was prepared by normal differential centrifugation procedures and the pellet was resuspended in a small volume of SET buffer.

Fluorescence Displacement Assays for Phospholipases A_2. Enzyme activity was assayed by a continuous fluorescence displacement assay in which the released long-chain fatty acid displaces the fluorescent probe, DAUDA, from recombinant rat liver FABP and the initial rate of loss of fluorescence is monitored (*14*, *28*). A typical assay contained phospholipid (50 μg/mL), 1 μM DAUDA, and 10 μg/mL FABP in 0.1 M Tris·HCl buffer (pH 8) with 2.5 mM $CaCl_2$. Calibration is achieved by adding oleic acid to the complete system in the absence of enzyme. Comparisons between different enzyme samples were always performed from the same preparation of substrate vesicles.

To monitor rat liver membrane hydrolysis, membrane fractions were first calibrated in terms of maximal releasable fatty acid. This was achieved by measuring total fatty acid release following hydrolysis by excess *N. naja* $sPLA_2$ by the fluorescence displacement assay. After calibration, 0.32 nmol of hydrolyzable phospholipid was diluted into 1 mL of HBSS containing 1 mM $CaCl_2$. DAUDA (1 μM) and FABP (10 μg) were added and hydrolysis was started by

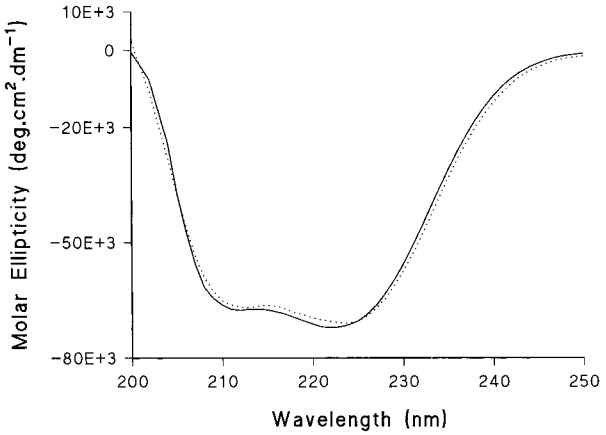

FIGURE 1: CD spectra of human $sPLA_2$ and the V3W mutants of $hnpsPLA_2$. The enzyme concentrations were 40 μM in 10 mM phosphate buffer, pH 7.4. The solid line is human $sPLA_2$ and the dotted line is the V3W mutant.

addition of the appropriate $sPLA_2$. It should be noted that membrane preparations are not very sensitive in terms of monitoring fatty acid release by the fluorescence assay due to the presence of significant amounts of endogenous free fatty acids. These fatty acids, which significantly reduce the fluorescence change per mole of released fatty acids, necessitate the use of small amounts of membranes in these assays. All assays were performed at 37 °C.

RAW 264.7 cell hydrolysis was followed by the fluorescence displacement assay as described elsewhere (*14*). Similar results were obtained with 3T3-L1 fibroblasts (not shown).

Radiochemical Assay for Human $sPLA_2$. Standard fluorescence assays were performed in which the DOPC contained 2000 dpm/nmol 1,2-di[1-^{14}C]oleoyl]PC. The assays were monitored for 5 min and the reaction was terminated by addition to 2 mL of chloroform/methanol (1:1 v/v). After addition of oleic acid (10 μg), the extracted lipids were separated by TLC with chloroform/methanol/acetic acid/water (25:15:4:2 v/v/v/v) as developing solvent. The radioactivity was determined by scintillation counting.

RESULTS

General Properties of V3W Mutant. The preparation and some basic properties of the V3W mutant of human $sPLA_2$ have been described (22). The mutation was confirmed both by N-terminal sequencing of human $sPLA_2$ and V3W proteins and by electrospray mass spectrometry, which gave molecular masses that differed by 86 mass units against a predicted difference of 87 mass units for a V to W mutation. The CD spectra of human $sPLA_2$ and the V3W mutant (Figure 1) indicated no significant differences in the gross structure of the two proteins consistent with the very similar catalytic properties when assayed under scooting mode conditions, as detailed below. Since it is apparent from its catalytic properties that the V3W mutant must be structurally very similar if not identical to recombinant human $sPLA_2$, no further detailed studies of the physical properties of this mutant were performed.

Phospholipid Vesicle Hydrolysis by the V3W Mutant. The assay of human $sPLA_2$ and the V3W mutant was performed with different phospholipid substrates presented as vesicles prepared by the solvent injection method. A continuous fluorescence displacement assay that measures fatty acid release (*27*) was used, and this assay allows comparison of phospholipid substrates that are not available in a radiochemically labeled form. These assays were performed with the anionic phospholipids DOPG and DOPM, both of which are good substrates for human $sPLA_2$. In addition, the zwitterionic phospholipids DOPC or egg PC were used, as these zwitterionic phospholipids are extremely poor substrates for human $sPLA_2$ (*13*, *14*, *22*), due to the enzyme being unable to bind productively to the PC interface. The specific activities were determined from the initial rates of hydrolysis for fixed concentrations of substrate, and comparison between enzymes involved assaying samples from the same vesicle preparation of each substrate. This precaution will minimize any variation in the interfacial quality of substrate presented to individual enzymes. Because the V3W mutation will produce an enzyme that resembles more closely the pancreatic enzyme, which already has a unique tryptophan at position 3, the specific activities of the porcine pancreatic $sPLA_2$ were also determined under these assay conditions for comparative purposes.

Table 1: Hydrolysis of Single Phospholipid Substrates by Secreted Phospholipases A_2[a]

	specific activity (μmol min^{-1} mg^{-1})			
type of $sPLA_2$	DOPG as substrate	DOPM as substrate	DOPC as substrate	egg PC as substrate
V3W mutant	59 ± 4	27 ± 1	10 ± 0.4	1.1 ± 0.07
human $sPLA_2$	107 ± 6	86 ± 4	0.01 ± 0.004	<0.01[b]
porcine pancreatic $sPLA_2$	210 ± 21	428 ± 38	66 ± 7	13 ± 0.6

[a] PLA_2 assays were performed in the presence of 2.5 mM $CaCl_2$ with single phospholipid substrates (50 μg/mL) presented as SUVs by solvent injection, as described in Materials and Methods. Specific activities were derived from the initial rates of hydrolysis obtained under these assay conditions. Results shown are the mean ± standard deviation of three determinations. [b] Activity with this substrate was below the level of detection of the assay under the conditions employed.

The results of specific activity measurements are shown in Table 1. They highlight a dramatic 1000-fold enhancement in activity resulting from the V3W mutation on both DOPC and eggPC when compared with the almost zero activity seen with human $sPLA_2$ and these substrates. In contrast, the specific activity of the V3W achieved when assayed with two anionic phospholipids, DOPM and DOPG, was routinely 25−50% that seen with human $sPLA_2$. For comparison, the porcine pancreatic enzyme expressed significant activity with PC vesicles, while the pancreatic enzyme expressed greater activity with DOPM compared with DOPG vesicles, whereas the reverse was the case with the human enzyme.

The validity of the assay in terms of response to enzyme concentration is shown in Figure 2, where a linear response with 50 μg/mL DOPC as substrate is observed over the range 50−1000 ng/mL for the V3W enzyme. Similarly, the initial rate with the wild-type enzyme increased over the range 5−27.5 μg/mL, although the larger error at high concentrations may reflect the very high concentration of enzyme being used relative to substrate. The DOPC:$sPLA_2$ molar ratio is 32:1 at 27.5 μg/mL enzyme, and at this ratio the interfacial surface and hence substrate concentration could become a limiting feature of the assay.

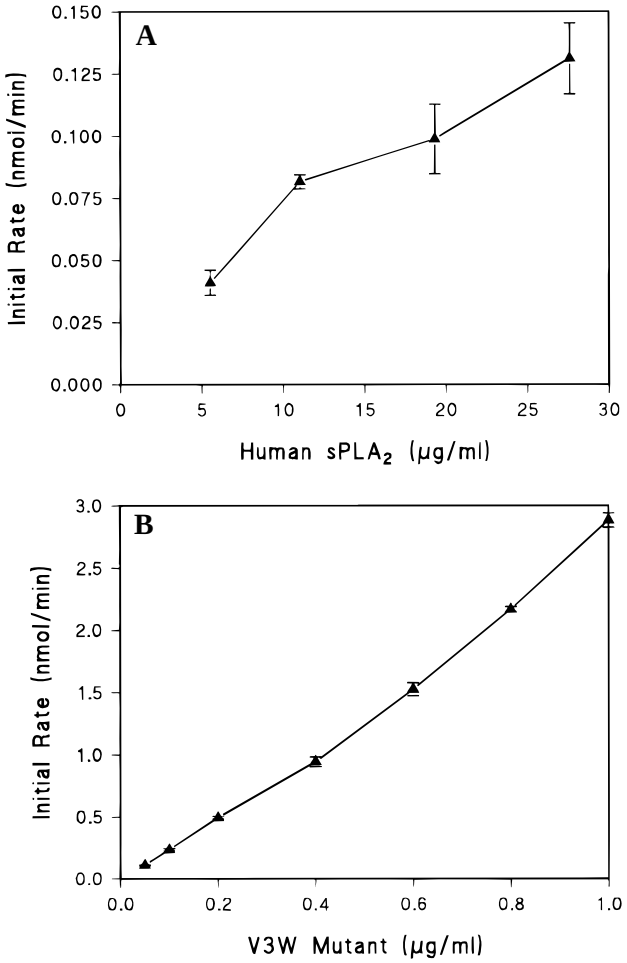

FIGURE 2: Effect of $sPLA_2$ concentration on the initial rate of hydrolysis of DOPC vesicles. Fluorescence assays contained 50 μg/mL DOPC and were performed as described in Materials and Methods. The fall in fluorescence was calibrated by addition of up to 2 nmol of oleic acid (0.2 mM in methanol) to an assay in the absence of enzyme. (A) Human $sPLA_2$; (B) V3W mutant.

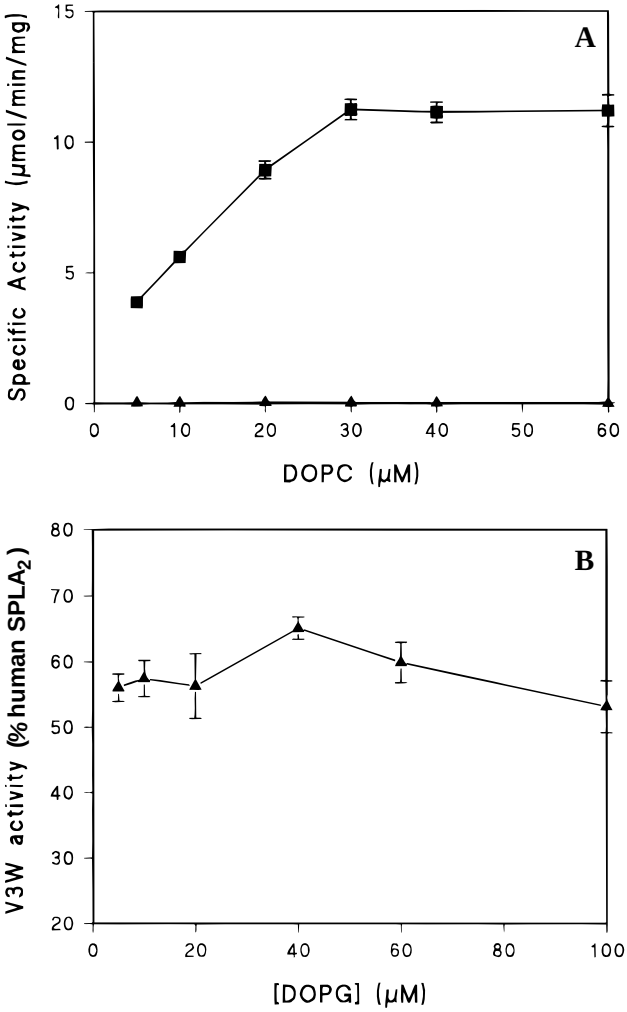

FIGURE 3: Effect of substrate concentration on the rate hydrolysis by human $sPLA_2$ and the V3W mutant. Fluorescence assays contained the appropriate amount of phospholipid and were performed as described in Materials and Methods. (A) Assays with DOPC involved 1.1 μg/mL of the human $sPLA_2$ and 0.6 μg/mL of the V3W mutant. (●) Human $sPLA_2$; (▲) V3W mutant. (B) Assays with DOPG involved 20 ng/mL of human $sPLA_2$ and 40 ng/mL of the V3W mutant. The activity of the V3W mutant is plotted as a percentage of the human $sPLA_2$ activity.

When the DOPC concentration was varied, the V3W showed substrate saturation above 30 μM DOPC, whereas the recombinant human $sPLA_2$ activity remained negligible (Figure 3) at the concentration of enzyme used for the comparison. Therefore, the difference in rate of the two enzymes cannot be overcome by using higher concentrations of substrate. Because of significant partitioning of DAUDA into neutral PC vesicles resulting in loss of assay sensitivity, higher concentrations of DOPC cannot be used reliably with this fluorescence assay. The problem of partitioning of the anionic DAUDA is reduced when anionic phospholipid vesicles such as those derived from DOPG are used.

To confirm that the comparison of rates with DOPG as substrate were being made under scooting conditions, where the effect of interfacial binding is minimized (*29*), rates were compared over the DOPG concentration range 5−100 μM. The rate at 100 μM DOPG was very similar to that seen at 5 μM DOPG, consistent with hydrolysis under scooting conditions. Moreover, as seen in Figure 3, the V3W mutant rate was maintained at 50−60% of the human $sPLA_2$ rate over this range of substrate concentration.

The enhancement of activity with the V3W mutant and PC vesicles is the most dramatic recorded for PLA_2 mutagenesis, the degree of stimulation being magnified by the negligible activity of human $sPLA_2$ with this substrate. To show a direct visual comparison of the initial rates of the two enzymes in real time using the fluorescence assay, it was necessary to use disparate amounts of the two enzymes. Comparison of 12.4 μg/mL human $sPLA_2$ with 100 ng/mL V3W mutant is illustrated in Figure 4 and shows that the rate was approximately double for V3W. This comparison corresponded to specific activities of 0.021 ± 0.002 and 5.3 ± 0.3 μmol min^{-1} mg^{-1}, respectively, under these assay conditions and hence the rate enhancement of the V3W is over 250-fold. In general, the activity of the V3W mutant was enhanced 200−1000-fold compared to that of human $sPLA_2$ when assayed on SUVs of phosphatidylcholine. This variation reflected difficulties in measuring accurately the low rates of human $sPLA_2$ unless excessive amounts of enzyme were used.

The fluorescence displacement assay used in the above comparisons is an indirect measure of enzyme activity. To ensure that the large differences seen between the wild-type and V3W mutant when hydrolyzing PC substrates were real,

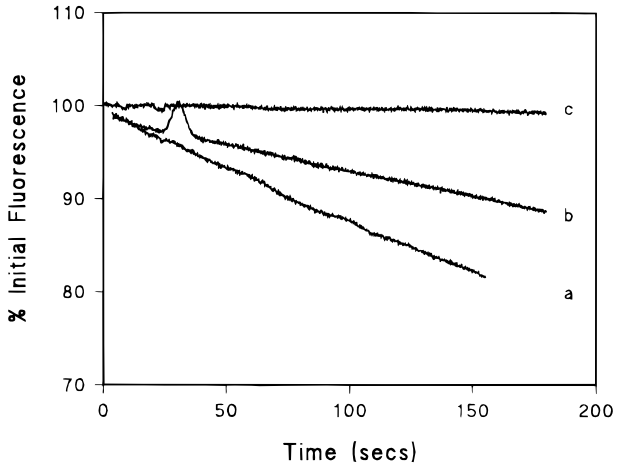

FIGURE 4: Fluorescence displacement traces for the hydrolysis of DOPC by human $sPLA_2$ and the V3W mutant. Fluorescence assays (1 mL) were performed as described for Figure 2. Enzyme was added at time zero and the actual real-time fluorescence traces are shown as released oleic acid displaces DAUDA from FABP. A 10% fall in fluorescence corresponds to the release of 0.5 nmol of oleic acid in these assays. The anomaly in trace b is due a dust particle in the light path. (a) V3W (100 ng/mL); (b) human $sPLA_2$ (12.4 μg/mL); (c) blank (no enzyme).

a comparison was performed with a radiochemical assay employing radioactive DOPC and involving measurement of fatty acid product after separation by TLC. The comparison required incubating either 5 μg of recombinant human $sPLA_2$ or 100 ng of V3W mutant for 5 min with 50 μg/mL DOPC, under which conditions the reaction was linear as monitored by the fluorescence assay. The net release of oleic acid was 0.26% $\pm$ 0.16% and 3.45% $\pm$ 1.0% of the total incubated radioactivity for the wild-type and mutant enzymes, respectively. This corresponds to an average specific rate enhancement of 663-fold for the V3W mutant and confirms the validity of the rate enhancement as measured by the fluorescence method.

Has the V3W Mutation Affected the Binding of PC to the Active Site of the Enzyme? The presumption so far has been that the V3W mutation affects the interfacial properties of the enzyme, that is, the $E \leftrightarrows E^*$ equilibrium where E* reflects the activated enzyme bound to the interfacial surface. To eliminate the effects of interfacial binding, assays were repeated under scooting conditions with nonhydrolyzable anionic vesicles prepared from DTPM and containing 4 mol % of a hydrolyzable phospholipid substrate. Under these conditions the enzyme is essentially irreversibly bound to the interface and assay rates observed will reflect the actual rate of substrate hydrolysis independent of interfacial binding (*29*). The results (Table 2) clearly show that under these scooting conditions the human $sPLA_2$ and the V3W mutant express similar activity with DOPC, the V3W rate being about 60% that of the human $sPLA_2$. This small reduction in specific activity with the V3W is similar to that seen when DOPG was the substrate (Table 1), although overall, the rates were at least 10-fold higher with DOPG than with DOPC. This preference for PG compared with PC under scooting conditions has already been observed for this human $sPLA_2$, using an alternative approach involving a technique employing polymerized liposomes (*23*). Overall, the data strongly suggest that the greatly enhanced activity of the V3W enzyme with zwitterionic vesicles is due to enhanced interfacial binding and activation, while substrate binding and hydrolysis at the active site are not affected.

Table 2: Comparison of the Hydrolysis by $SPLA_2$s of DOPG and DOPC Presented at 4 Mol % in a DTPM Interface[a]

	specific activity (μmol min^{-1} mg^{-1})		
type of $sPLA_2$	DTPM	4 mol % DOPC	4 mol% DOPG
V3W mutant	not detectable	1.05 ± 0.02	15 ± 3
human $sPLA_2$	not detectable	1.5 ± 0.1	22 ± 6

[a] Hydrolyzable phospholipid was mixed at 4 mol % with DTPM prior to MLV formation. SUVs were prepared from these MLVs by sonication. Enzyme activity was measured by the fluorescence displacement assay. Results shown are the mean $\pm$ standard deviation of three determinations.

Does the Presence of the Interfacial Tryptophan Enhance Hydrophobic Interactions? If the presence of the Trp-3 were enhancing binding by a hydrophobic contribution, resulting from bilayer penetration of the indole side chain, such a nonpolar interaction should be stabilized by increasing the salt concentration of the assay medium. In contrast, electrostatic interactions between the enzyme and the phospholipid interface are weakened by such conditions. When DOPG SUVs were used as substrate to allow comparison of the activities of human $sPLA_2$ and the V3W mutant, the activity of the mutant was significantly enhanced at a higher salt concentrations compared to that of the normal enzyme (Figure 5). This enhancement results in V3W expressing similar activity to human $sPLA_2$ at higher ionic strength and suggests that interfacial binding of these proteins is a subtle balance of hydrophobic and electrostatic interactions. The potential importance of hydrophobic interaction in the interfacial binding of $sPLA_2$ to anionic phospholipids has recently been highlighted in the case of bee venom (*30*). Assay of the V3W mutant with DOPC as substrate (Figure 5) clearly showed an inhibitory effect with increasing salt concentration. This at first sight is a surprising result if the tryptophan were playing a major role in interfacial binding. It is possible that interfacial binding to a zwitterionic interface is due to an ensemble of conformations (*31*) limited by an initial electrostatic encounter complex.

Hydrolysis of Biological Membranes by the V3W Mutant. Because the V3W mutant is able to hydrolyze DOPC SUVs, it was of interest to see if this enzyme was able to show enhanced hydrolysis of whole cells and biological membranes. We have previously shown that human $sPLA_2$ expresses very low activity with such membranes (*14*), whereas significant activity was detected in this fluorescence assay with porcine pancreatic enzyme. As a positive control in that study, the ability of the highly penetrating venom enzyme from *N. naja* to readily hydrolyze such membranes was clearly demonstrated.

The results of comparisons of human $sPLA_2$, pancreatic $sPLA_2$, and *N. naja* venom $sPLA_2$ with whole cells (macrophages) as substrate are shown in Table 3. All specific activities are low compared to those obtained with phospholipid vesicles; however, a 10-fold enhanced activity of the V3W mutant compared with human $sPLA_2$ is clearly seen and this activity with the V3W mutant is similar to that expressed with the porcine pancreatic enzyme using the same substrates. The considerable ability of the *N. naja* venom enzyme to hydrolyze whole cells is also demonstrated.

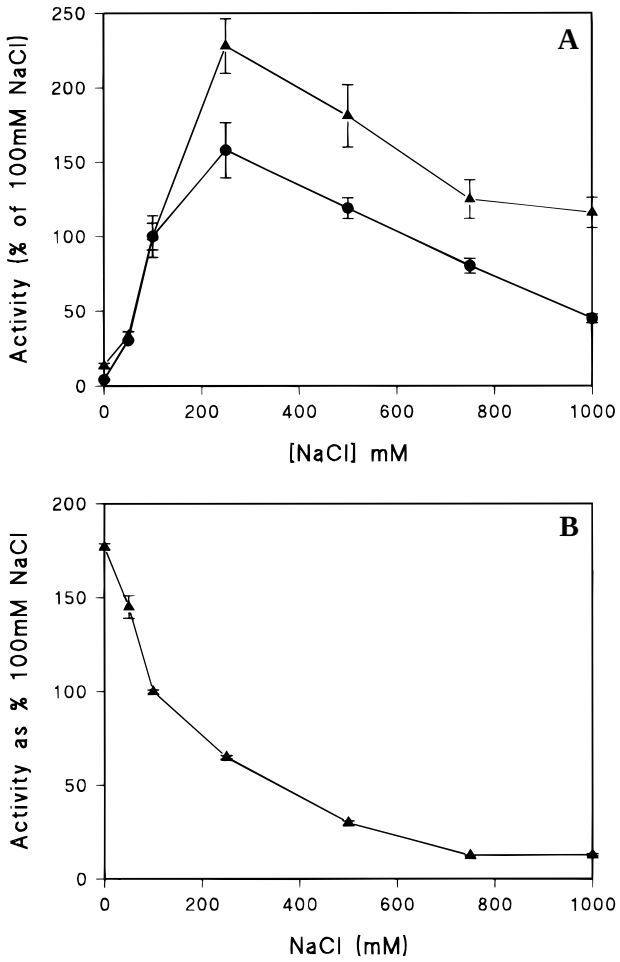

FIGURE 5: Effect of NaCl on the hydrolysis of phospholipid vesicles by human $sPLA_2$ and the V3W mutant. (A) Fluorescence assays (1 mL) contained 4 μg/mL DOPG in 10 mM Tris·HCl, pH 8.0, with 0.5 mM $CaCl_2$, 1 μM DAUDA, and FABP (10 μg/mL). Assays contained 30 ng/mL of human $sPLA_2$ and 60 ng/mL of the V3W mutant in the absence of NaCl. Enzyme concentrations were reduced where higher activities were being expressed. (●) Human $sPLA_2$; (▲) V3W mutant. (B) Fluorescence assays (1 mL) contained 50 μg/mL DOPC in 10 mM Tris·HCl, pH 8.0, with 0.5 mM $CaCl_2$, 1 μM DAUDA, and FABP (10 μg/mL). V3W was used at 0.6 μg/mL up to 500 mM NaCl and then at 1.2 μg/mL.

Table 3: Hydrolysis of Biological Membranes by Secreted Phospholipases A_2[a]

	specific activity (μmol min^{-1} mg^{-1})		
type of $sPLA_2$	RAW 264.7 cells	rat liver microsomes	rat liver plasma membranes
V3W mutant	0.302 ± 0.007	2.15 ± 0.12	3.34 ± 0.04
human $sPLA_2$	0.031 ± 0.002	0.06 ± 0.004	0.050 ± 0.003
porcine pancreatic $sPLA_2$	0.368 ± 0.002	0.606 ± 0.067	n.d.
Naja naja venom $sPLA_2$	9.67 ± 1.0	74 ± 5	19 ± 1.0

[a] All enzyme activities were measured by the fluorescence displacement assay as described in Materials and Methods. All assays were performed at 37 °C in HBSS with 1 mM $CaCl_2$. Assays involving whole cells as substrate contained 400 000 cells/mL for the RAW 264.7 cells. Assays involving biological membrane preparations contained 0.32 nmol/mL hydrolyzable phospholipid. nd, not determined. Results shown are the mean ± standard deviation of three determinations.

When this study of biological membrane hydrolysis was extended to rat liver microsomes and plasma membranes, a similar pattern was seen, although the difference in activities between the V3W and human $sPLA_2$ was accentuated compared to results with whole cells. It should be noted that such membrane fractions would be more highly curved than the plasma membrane of whole cells and this may explain the differential hydrolysis by the V3W mutant (see below).

How Does Vesicle Curvature Affect PC Hydrolysis by the V3W Mutant? It is well-established that both secreted and cytosolic PLA_2 express higher activity on small, highly curved vesicles of PC than on larger, more planar vesicles. This is because the less condensed nature of the more strained outer monolayer of the small vesicle facilitates enzyme interactions and substrate extraction.The low activity expressed with biological membranes, particularly whole cells, may be due in part to the more planar nature of these vesicles compared to the high curvature of SUVs. To determine the effect of vesicle curvature on the rate of phospholipid hydrolysis, MLVs prepared from DOPC were assayed as substrates and compared with SUVs prepared from these MLVs by probe sonication. Whereas both human $sPLA_2$ and the V3W mutant expressed negligible activity with MLV presentations of DOPC, sonication to generate SUVs resulted in a dramatic increase in activity of the V3W mutant to about 2 μmol min^{-1} mg^{-1} while, as expected, the activity of the human $sPLA_2$ remained negligible. Thus, the enhanced activity of the V3W mutant with zwitterionic interfaces is accentuated when that interface is more curved and hence of a less condensed nature, possibly facilitating insertion of the tryptophan side chain into the bilayer. We would argue that this physical phenomenon may make a major contribution to the lower activity of the V3W mutant expressed in the more planar biological membranes.

Is It Possible To Detect Enhanced Binding of the V3W Mutant to PC Interfaces? In an attempt to demonstrate enhanced binding of the V3W mutant to the PC interface compared with human $sPLA_2$, MLVs of DOPC were used, as these can be sedimented by centrifugation and enzyme binding can be monitored by assaying enzyme activity remaining in the supernatant after centrifugation. However, for both enzymes essentially 100% of enzyme activity was still detected in the supernatant after centrifugation and established that neither human $sPLA_2$ nor the V3W mutant bound to such vesicles. This result may be rationalized by the fact that only after sonication to produce SUVs (which cannot be sedimented by ultracentrifugation) is the V3W able to catalyze vesicle hydrolysis. The validity of the centrifugation method was confirmed by using MLVs prepared from DOPG when <0.1% of both enzymes remained in the supernatant after ultracentrifugation.

Protein Modeling. In view of the dramatic effect of the V3W mutation on the hydrolysis of PC vesicles, the tryptophan at position 3 was modeled into the structure of human $sPLA_2$ and this structure was compared with our recombinant human $sPLA_2$. A comparison with porcine pancreatic $sPLA_2$ is included because the V3W mutant is similar to it in terms of interfacial activity and also the three amino-terminal residues, ALW, are identical. The results of such modeling are shown in Figure 6 and clearly demonstrate the exposed position of the inserted tryptophan above the presumptive interfacial surface of the enzyme where it would be in an excellent position to intercalate into the bilayer, as seen with the pancreatic enzyme.

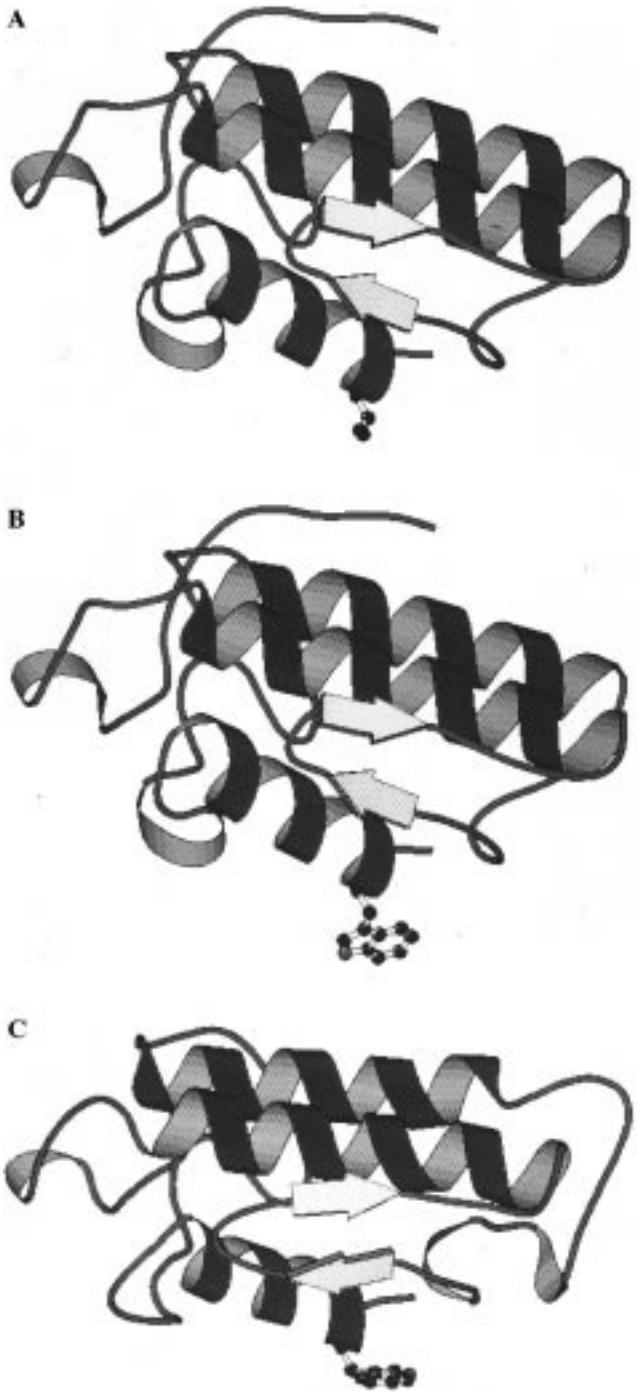

FIGURE 6: Ribbon diagrams of human $sPLA_2$, the V3W mutant, and porcine pancreatic $sPLA_2$, showing the position of Val-3, Trp-3, and Trp-3, respectively. All analysis and modeling of the V3W mutant were performed with the program package Quanta/CHARM. The figures were generated with Molscript (*42*) and show the amino acid residue at position 3. The crystal structure of the human $sPLA_2$ enzyme is from ref *43*, and for the porcine pancreatic enzyme, from ref *44*. In the orientation shown, the phospholipid interface is below the residue illustrated at position 3. (A) Recombinant human $sPLA_2$; (B) V3W mutant of human $sPLA_2$; (C) porcine pancreatic $sPLA_2$.

DISCUSSION

The phenomenon of interfacial activation is a characteristic of enzymes that act on an aggregated lipid substrate. The presumptive conformational change that must occur on interfacial binding to produce activation is clearly seen in the case of triglyceride lipase, where a repositioning of an active-site lid on binding allows direct access of the substrate to the active site (*32*). In the case of $sPLA_2$s, the amino-terminal residues have been the focus of attention. Fluorescence and NMR studies (*31*, *33*−*35*) have highlighted the mobility of Trp-3 of the porcine pancreatic $sPLA_2$ despite the apparent α-helical organization of this region seen in a variety of crystal structures of mammalian and venom enzymes. Recent detailed NMR studes of the pancreatic enzyme have demonstrated the mobility of the first three amino-terminal residues, ALW, of the free enzyme; however, when the enzyme is bound to an interface, these residues adopt a fixed conformation similar to that seen in the crystal structure (*36*, *37*). These observations provided the first direct evidence of a conformational change within the $sPLA_2$ on interfacial binding that could produce interfacial activation and highlight the involvement of Trp-3 in the process.

In the case of the bovine pancreatic enzyme, mutating of this tryptophan to an alanine (W3A) produced an enzyme that displayed only modest changes in its catalytic properties. However, binding to anionic vesicles had been sufficiently perturbed so that hopping between vesicles rather than the normal scooting-mode kinetics was observed (*21*). Of particular relevance to the present work are mutational studies on the bovine pancreatic enzyme where the change of the leucine at position 20 to a tryptophan (L20W) resulted in an enzyme with enhanced interfacial binding to densely packed neutral monolayers and bilayers (*18*). This mutation is of particular significance because the *N. naja* venom $sPLA_2$ has high activity toward zwitterionic bilayers including cell membranes (*14*, *16*) and has a tryptophan at this position.

The amphiphilic indole side chain of tryptophan has been shown to partition at the lipid−water interface (*19*) and an increasing number of proteins that bind to the membrane surface, particularly a zwitterionic interface, are recognized to involve tryptophans. For example, the enzyme lipoprotein lipase has a cluster of three tryptophans on the presumptive interfacial surface, and mutations of some of these residues greatly reduces the ability of the enzyme to bind to and hydrolyze chylomicrons but not monomeric substrates (*38*). Recently, the human secretory group V phospholipase A_2 has been expressed in *E. coli* (*39*); it contains four tryptophan residues and this enzyme can express high activity with a PC interface (*39*).

In view of the potentially important role of a tryptophan residue in binding a protein to a lipid−water interface, the lack of such a tryptophan in human $sPLA_2$ could well contribute to the negligible activity of this enzyme with zwitterionic interfaces, particularly when compared to the activity of pancreatic $sPLA_2$ on such interfaces. If this were the case, then the production of a V3W mutant in which the crucial terminal three residues, ALW, are now identical to that of the porcine (and human) pancreatic enzyme was of considerable interest. This V3W mutant of the human $sPLA_2$ was slightly less active with anionic substrates such as DOPG or DOPM, the activity being about 25−50% that of human $sPLA_2$ under normal assay conditions. However, a dramatic difference in activity was seen with PC as substrate. Although the activity of both the human $sPLA_2$ and V3W was negligible with MLVs of low curvature, when these MLVs where converted into SUVs by sonication a large increase in activity was seen with the V3W that was not seen with human $sPLA_2$. Hence the V3W mutant is able to express low but significant activity with such vesicles, and

since the activity of the human $sPLA_2$ on this substrate is virtually zero, an enhancement in activity of the order of 1000-fold is possible.

The enhanced activity of the V3W mutant may be one of the largest activitity enhancements seen in the field of enzyme engineering and reflects the major contribution that interfacial binding can make to the overall catalytic activity of surface-acting enzymes. That this enhanced activity was due to enhanced binding at the interface and not to a change in catalytic events at the active site was confirmed by assaying PC hydrolysis under scooting conditions (*29*), where high-affinity interfacial binding is assured. Under these conditions both the human $sPLA_2$ and the V3W mutant expressed significant activity, with the mutant having about 60% the activity of the human $sPLA_2$.

We have previously shown that the human enzyme expresses negligible activity toward whole cells, with cell membrane hydrolysis being significant at enzyme concentrations above 1 μg/mL (*14*). In this study we can demonstrate that the V3W is about 10-fold more active with such whole-cell membrane presentations, which will be of low curvature. Similarly, enhanced activity of the V3W was seen with both rat liver microsomes and plasma membranes, the rates now being about 100-fold higher than the wild-type enzyme and possibly reflecting the increased curvature of such membrane preparations.

Molecular modeling has confirmed the availability of the tryptophan introduced at position 3 to participate in interfacial binding, while molecular dynamic studies of human $sPLA_2$ have identified a number of nonpolar residues including Leu-2, Val-3, Ala-18, Leu-19, Phe-24, Val-31, and Phe-70 as interacting with the lipid surface (*40*). Recent studies involving the bee venom $sPLA_2$ have attempted to define in more detail the topological relationship between membrane and protein (*41*), while the importance of nonpolar contributions to the interaction has been highlighted (*30*). Our understanding of the molecular interactions involved in the binding of enzymes to the membrane interface is still incomplete, particularly the relative roles of polar and nonpolar interactions.

In conclusion, the mutation of the interfacial valine of human $sPLA_2$ to a tryptophan has dramatically highlighted the ability of this amphiphilic indole residue to greatly facilitate the hydrolysis of zwitterionic interfaces presented in the form of SUVs of PC or cell membranes. These results provide strong support for the role of interfacial tryptophans of venom enzymes in allowing the penetration and hydrolysis of such condensed bilayers. In general terms, tryptophans at the interface facilitate the penetration and hydrolysis of PC monolayers and bilayers that have high surface pressures. It is important for the human $sPLA_2$, which can be present at high concentration in the serum and other extracellular fluids, probably as an acute-phase protein, to lack this penetrating ability; otherwise the enzyme would hydrolyze both normal cells and lipoproteins. Presumably, the human group IIa enzyme evolved without an interfacial tryptophan to minimize normal cell hydrolysis by this extracellular enzyme.

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