

Neurotoxicity and Other Pharmacological Activities of the Snake Venom Phospholipase A₂ OS₂: The N-Terminal Region Is More Important Than Enzymatic Activity[†]

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ABSTRACT: Several snake venom secreted phospholipases A₂ (sPLA₂s) including OS₂ exert a variety of pharmacological effects ranging from central neurotoxicity to anti-HIV activity by mechanisms that are not yet fully understood. To conclusively address the role of enzymatic activity and map the key structural elements of OS₂ responsible for its pharmacological properties, we have prepared single point OS₂ mutants at the catalytic site and large chimeras between OS₂ and OS₁, a homologous but nontoxic sPLA₂. Most importantly, we found that the enzymatic activity of the active site mutant H48Q is 500-fold lower than that of the wild-type protein, while central neurotoxicity is only 16-fold lower, providing convincing evidence that catalytic activity is at most a minor factor that determines central neurotoxicity. The chimera approach has identified the N-terminal region (residues 1–22) of OS₂, but not the central one (residues 58–89), as crucial for both enzymatic activity and pharmacological effects. The C-terminal region of OS₂ (residues 102–119) was found to be critical for enzymatic activity, but not for central neurotoxicity and anti-HIV activity, allowing us to further dissociate enzymatic activity and pharmacological effects. Finally, direct binding studies with the C-terminal chimera, which poorly binds to phospholipids while it is still neurotoxic, led to the identification of a subset of brain N-type receptors which may be directly involved in central neurotoxicity.

Secreted phospholipases A₂ (sPLA₂s)¹ comprise a large family of structurally conserved enzymes that catalyze the hydrolysis of glycerophospholipids at the *sn*-2 position to release free fatty acids and lysophospholipids. These enzymes were first discovered in insect and snake venoms, and subsequently in mammalian tissues, plants, bacteria, fungi,

and viruses (1–6). Most sPLA₂s share a common set of structural features including a relatively small molecular mass (14–19 kDa), a compact structure with several disulfides, and a conserved Ca²⁺-dependent catalytic mechanism.

To date, up to 12 different sPLA₂s that belong to three main structural collections have been identified in mammals (1, 7–9). Despite an important knowledge accumulated at the molecular level (1, 9–11), the exact biological functions of most mammalian sPLA₂s remain to be elucidated. Known functions include lipid digestion, host defense, and lipid mediator production during normal and inflammatory processes. Some of these biological roles have been attributed to the first identified sPLA₂s, namely, the group IB, IIA, V, and X enzymes. Almost nothing is known about the

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¹ Abbreviations: ACN, acetonitrile; CD, circular dichroism; DSS, Suberic acid bis-*N*-hydroxysuccinimide ester; HIV-1, human immunodeficiency virus-1; LD₁₀₀, lethal dose for 100% of the population tested; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; OS₁, *Oxyuranus scutellatus scutellatus* toxin 1; OS₂, *Oxyuranus scutellatus scutellatus* toxin 2; recOS₂, recombinant OS₂; vOS₂, venom OS₂; POPG/S/C, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol/serine/choline; sPLA₂, secreted phospholipase A₂; TFA, trifluoroacetic acid; WT, Wild-Type.

biological functions of the more recently identified sPLA₂s IID, IIE, IIF, III, XIA, and XIIB (8, 12).

Much more is known about the pharmacological effects of snake venom sPLA₂s. More than 280 venom enzymes have been reported over several decades (http://sdmc.lit.org.sg/Templar/DB/snaketoxin_PLA2/index.html), and many of them display a wide spectrum of toxic effects including neurotoxic, myotoxic, cardiotoxic, cytotoxic, anticoagulant, convulsant, hypotensive, and pro-inflammatory effects (2, 13–16). In vitro, they can also modulate cell migration and cell proliferation (17, 18), have antibacterial activity (19), and display potent inhibitory effects against HIV-1 (20) and *Plasmodium falciparum*, the most deadly malaria parasite (21). Because of this tremendous array of pharmacological effects, it is possible that mammalian sPLA₂s may exert much more diverse functions than those currently known (22).

Although many venom sPLA₂s share similar structural features and catalytic activity, not all venom enzymes exhibit all of the above pharmacological effects (2). Furthermore, some venom sPLA₂s have no catalytic activity, while they exert various toxic and pharmacological effects (14, 16, 18–20). The absence of direct correlation between catalytic activity and pharmacological effects has led to the hypothesis that the specific actions of sPLA₂ are due to the presence of pharmacological sites on the sPLA₂ surface which are separated from or overlap with the catalytic site. These pharmacological sites would allow the sPLA₂ to bind specifically to soluble proteins or membrane-bound proteins that participate in the sPLA₂ mechanism of action (23, 24). Since this hypothesis was proposed, a collection of binding proteins has been identified using several toxic snake venom sPLA₂s (25–28). Besides β -bungarotoxin (29), early studies with the neurotoxic sPLA₂ OS₂ from the Australian Taipan snake *Oxyuranus scutellatus scutellatus* have led to the identification of two families of binding proteins called N- and M-type receptors (25, 30). The N-type receptors bind OS₂ with picomolar affinities, are present in mammalian brain and other tissues, and are made up of multiple proteins with molecular masses of 18–24, 36–51, and 85 kDa. Other neurotoxic sPLA₂s bind to the N-type receptors with high affinities, while nontoxic sPLA₂s including OS₁ bind with much lower affinities, suggesting that these receptors are involved in sPLA₂ central neurotoxicity. Conversely, the M-type receptor, which binds with high affinity both toxic and nontoxic sPLA₂s including OS₂ and OS₁, consists of a single protein of 180 kDa and belongs to the C-type lectin superfamily (25). Importantly, the M-type receptor binds several mammalian sPLA₂s (31, 32), suggesting that these proteins are the endogenous ligands for this receptor, and possibly for the collection of binding proteins initially identified with venom sPLA₂s. Binding studies with ammodytoxins from the long-nosed viper *Vipera ammodytes ammodytes* have also led to the identification and characterization of several proteins in the brain that appear related to N- and M-type receptors ((27) and references therein).

Despite the identification of the above binding targets and many structure–function studies based on amino acid modification, site-directed mutagenesis, and structural comparison (33–36), the molecular mechanisms underlying many of the pharmacological actions of venom sPLA₂s are not yet fully understood (2, 14, 37, 38). In particular, the neurotoxicity of venom sPLA₂s, which has been one of the

most studied pharmacological effects, is currently thought to involve both catalytic activity and binding to target proteins on presynaptic membranes (39, 40). The assumption that enzymatic activity is required for neurotoxicity is however essentially based on old studies showing that alkylation of the active site histidine of several neurotoxic sPLA₂s by *p*-bromo-phenacyl-bromide substantially decreases their neurotoxicity (41–43). However, there was a greater loss of enzymatic activity than neurotoxicity, suggesting that other factors must also contribute to neurotoxicity. The fact that alkylation of different sPLA₂s by *p*-bromo-phenacyl-bromide leads to conformational changes at the surface of the enzyme (44–46) raises the possibility that the loss of neurotoxicity may not be due to a decrease of catalytic activity per se, but rather to an altered ability of the enzyme to interact with high affinity with its specific binding protein target on neuronal membranes. On the other hand, significant efforts have been made to identify the sPLA₂ pharmacological site involved in neurotoxicity, but its exact location still remains ill-defined (2, 33, 35, 47). In the case of ammodytoxins, a series of structure–function studies have shown that residues located at both the N-terminal and C-terminal regions of these sPLA₂s are important for central neurotoxicity ((47) and references therein).

As indicated above, OS₂ has initially served as a key tool to identify the N- and M-type sPLA₂ receptors (25, 30). It is highly neurotoxic in mice and rats (30, 48), blocks acetylcholine release in *Aplysia* neurons (49), triggers cell migration (17), and potentiates proinflammatory cellular signaling (50), and we report here that it can also affect chick neuromuscular transmission, be myotoxic, and exert anti-HIV and antimalarial activities in vitro. The main purpose of this work was thus to determine more conclusively the role of catalytic activity, to identify the location of the OS₂ pharmacological sites responsible for central neurotoxicity and other pharmacological effects, and to analyze the relationship with M-type and N-type receptors. Based on the accumulated knowledge on sPLA₂ catalytic activity and the complexity of the reaction (51), we reasoned that the best way to probe the role of enzymatic activity was to produce OS₂ derivatives with single point mutations in the active site, rather than on the interfacial binding surface of the sPLA₂. To determine the location of the pharmacological sites on OS₂ and explain the large difference in neurotoxicity between OS₂ and the structurally related OS₁, we prepared large chimeras between the two proteins by grafting the divergent N-terminal, central, and C-terminal regions of OS₁ onto the OS₂ structure.

EXPERIMENTAL PROCEDURES

Construction of the OS₂ Synthetic Gene and Mutants. A number of considerations have been taken into account for the production of OS₂. First, as we could not obtain mRNA from the venom gland of the Australian Taipan snake, we constructed a synthetic gene for OS₂ based on the sequence of the venom protein (52). Second, since our experience indicates that some sPLA₂s cannot be easily refolded when produced in *Escherichia coli* while they can be obtained in a native form from insect cell systems and vice versa (11, 31), we tried expression of OS₂ in both S2 *Drosophila* cells and *E. coli*. Finally, since we found that the use of *E. coli*-preferred codons is not critical when the sPLA₂ is fused to

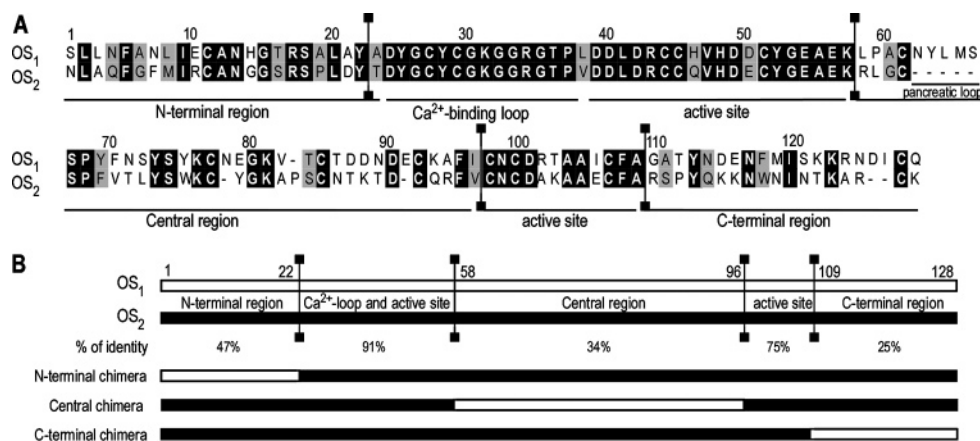


FIGURE 1: Alignment of the amino acid sequences of OS₁ and OS₂ and schematic representation of the chimeras between OS₁ and OS₂. (A) Alignment of the amino acid sequences of OS₁ and OS₂ (52). (B) Schematic representation of the chimeras between OS₁ and OS₂. The conserved and divergent regions between OS₁ and OS₂ are separated by vertical lines in panels A and B. The percentage of identity between the different regions are indicated. The N-terminal (1–22), central (58–96), and C-terminal (109–128) regions of OS₁ have been introduced into the OS₂ sequence to construct N-terminal, central, and C-terminal chimeras, respectively.

the N-terminal sequence of proteins such as glutathione S-transferase or thioredoxin (unpublished data and (53)), we designed the OS₂ synthetic gene using *Drosophila* preferential codons (54) that should allow expression in both insect cells and *E. coli*. The synthetic cDNA sequence (available on request) was designed using the backtranslate program from the Genetic Computer Group and assembled from three sets of six partially overlapping oligonucleotides (40-mer) in a three-step PCR process using Dynazyme polymerase (Finnzyme laboratories). In the first round of PCR (final volume 20 μ L), each set of oligonucleotides (5 μ g each, 3 forward and 3 reverse, 20-mer complementary sequences) was annealed (60 °C/20 s), elongated (72 °C/20 s), and denatured (94 °C/20 s) for 25 cycles. The DNA assemblies were further amplified in a second round of PCR (25 cycles, each 94 °C/20 s, 60 °C/20 s, 72 °C/20 s) using the two external oligonucleotides. The three PCR products were purified on agarose gel and used for a last round of PCR using the two oligonucleotides flanking the full OS₂ synthetic gene. The final PCR product was tailed with *Taq* polymerase, subcloned into the pGEM-T easy vector (Promega Corp.), and sequenced. The OS₂ cDNA was subcloned in frame into the S2 expression vector harboring the human group IIA sPLA₂ signal peptide or into the pAB₃ vector (11). Point mutants were prepared in a two-step PCR standard procedure (55) using complementary primers containing the mutation and primers flanking the OS₂ synthetic gene inserted into the pAB₃-OS₂ vector. Chimeras were constructed by inserting by PCR synthetic DNA fragments coding for the N-terminal, central, and C-terminal regions of OS₁ (Figure 1) into the pAB₃-OS₂ construction. All the final PCR products were entirely sequenced.

Production of Recombinant OS₂ Mutants and Chimeras in *Drosophila* S2 Cells and *E. coli*. Initial attempts to express OS₂ in *Drosophila* S2 cells were unsuccessful, although we used a protocol similar to the one used for several mouse and human sPLA₂s (11). Conversely, wild-type (WT) OS₂, single point mutants and chimeras could be produced in *E. coli* using the pAB₃ vector (11). The sPLA₂s were produced as insoluble C-terminal fusion proteins with the truncated glutathione S-transferase (8.7 kDa) and the factor Xa/trypsin proteolytic site (Ile-Glu-Gly-Arg) located before the N-terminal residue of the mature sPLA₂. Expression of fusion

proteins as inclusion bodies in *E. coli* BL21 cells and sulfonation from 100 mg of protein were performed as described (56). For refolding, the sulfonated protein was dissolved in 500 mL of 6 M guanidine-HCl and 50 mM Tris-HCl, pH 8.0, and dialyzed (8 kDa membranes, Spectrum laboratories) against 8 L of 0.7 M guanidine-HCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, and 5 mM L-cysteine (5 mM L-methionine was added for the central and C-terminal chimeras to prevent methionine oxidation) for 48 h at 4 °C. For the N-terminal chimera, the sulfonated protein was resuspended in 500 mL of 8 M urea, 0.1 M NH₄Cl, and 50 mM Tris-HCl, pH 8.0, and refolded by dialysis against 8 L of 1.6 M urea, 0.1 M NH₄Cl, 50 mM Tris-HCl, pH 8.0, 5 mM CaCl₂, and 5 mM L-cysteine for 48 h at 4 °C. The refolded protein was then dialyzed against 8 L of 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 1 mM CaCl₂ and clarified by centrifugation (10 000g, 30 min). The protein solution was subjected to trypsin digestion (1:30 trypsin/protein) which was monitored online by *E. coli* enzymatic assays (11) and mass spectrometry, and the reaction was stopped by adding 1% acetic acid and 1 mM phenylmethylsulfonyl fluoride. The digested protein was concentrated to 20 mL by ultrafiltration using an Amicon stirred cell concentrator with a YM10 membrane, and the buffer was exchanged to 1% acetic acid/10% acetonitrile (ACN). The refolded protein was purified sequentially by cation exchange HPLC on a Spherogel TSK SP-5PW column (3.3 mL, 10 μ m, 7.5 \times 75 mm, Tosoh Biosep, 1 mL/min, linear gradient of ammonium acetate (0–1 M, pH 6.8, over 50 min) in 10% ACN) followed by C18 reverse-phase HPLC (19.3 mL, 10 \times 250 mm, 100 Å, 5 μ m, Beckman, 4 mL/min, linear gradient of ACN in 0.1% TFA (10–28% ACN over 18 min, 28–35% ACN over 42 min)). The lyophilized proteins were quantified by OD_{280nm} using their calculated molar absorption coefficients and analyzed by MALDI-TOF mass spectrometry, SDS–polyacrylamide gel electrophoresis, and circular dichroism (CD). MALDI-TOF analysis of sPLA₂s was carried out on an Applied Biosystems voyager DE-PRO mass spectrometer in linear mode with sinapinic acid as a matrix, using external calibration. The secondary structure of all proteins was evaluated by CD using a Jasco PTC-423S (J-810) apparatus and analyzed with the program CDNN (CD Spectra deconvolution, version 2.1). Proteins were diluted

to 0.1 or 0.2 mg/mL in 10 mM Na⁺-borate, pH 8.0, and 0.1 mM EDTA, and spectra were recorded at 20 °C with a scanning speed of 20 nm/min between 190 and 260 nm. The final CD spectrum was obtained from the average of 15 runs.

Enzymatic Activity and Interfacial Binding to Phospholipid Vesicles. *E. coli* enzymatic assays were performed using [³H]-oleate-radiolabeled membranes (11). The headgroup substrate specificity was measured on large unilamellar vesicles of pure POPG, POPS, or POPC with the fatty acid-binding protein assay as described (11). The initial velocities were measured at 37 °C in 1.3 mL of Hanks' balanced salt solution containing 1.3 mM Ca²⁺, 0.9 mM Mg²⁺, 30 μM phospholipid-extruded vesicles, 1 μM 11-dansyl-undecanoic acid, and 10 μg of rat liver fatty acid binding protein. Excitation was at 350 nm and emission at 500 nm. Assays were calibrated by adding known amounts of oleic acid and measuring the decrease in fluorescence. Reactions were started by adding sPLA₂. Binding studies of sPLA₂s to sucrose-loaded, 0.1 μm unilamellar vesicles of diether phospholipids (20 mol % DOetPS/80 mol % DOetPC) were performed as described previously (11). sPLA₂ binding studies were carried out in 5 mM MOPS, pH 7.4, 0.1 M KCl, and 2 mM CaCl₂ at room temperature using the centrifugation method in which the amount of enzyme remaining in the supernatant of pelleted vesicles above-mentioned is quantified by enzymatic assays (for high specific activity enzymes) or silver-stained 16% SDS–PAGE gels (for both low- and high-specific activity enzymes). Assays were made in the presence of 0.5 μg of sPLA₂ and different concentrations of phospholipid vesicles in a total volume of 100 μL to measure the affinity (*K_d*) of the sPLA₂ for the phospholipid vesicle.

Receptor Binding Assays and Cross-Linking Studies. Competition binding assays between iodinated OS₂ and unlabeled OS₂ mutants on N-type and M-type receptors were performed under equilibrium binding conditions as described (20, 30, 31), except that OS₂ was labeled to a specific activity of 3000–3500 cpm/fmol using lactoperoxidase as follows. The iodination was performed in a total volume of 60 μL of 0.1 M NaH₂PO₄, pH 6.5, containing 0.5 nmol OS₂, 0.5 nmol ¹²⁵I-Na (Perkin-Elmer), 0.5 μg of lactoperoxidase (Sigma L8257), and 20 μL of hydrogen peroxide (Sigma H1009) diluted 1/40 000 in H₂O. The reaction was initiated by adding 10 μL of hydrogen peroxide at 0 and 10 min. After incubation at room temperature for 20 min, the mixture was diluted to 1 mL with H₂O/ACN/TFA (90/10/0.1) and applied to a C18 end-capped reverse-phase HPLC column (Merck purosphere STAR, 55 × 4 mm, 3 μm, 100 Å, bed volume 0.7 mL). Elution was performed at 1 mL/min using a linear gradient of ACN in 0.1% TFA (10–25% ACN over 10 min, 25–50% ACN over 50 min). Fractions containing active iodinated OS₂ were identified by binding assays on M- and N-type receptors. Binding on M-type receptors was carried out on membranes from HEK 293 cells (ATCC CRL-1573) transfected with either rabbit- or mouse-cloned M-type receptors (31, 57). Binding on N-type receptors was carried out on mouse brain membranes (30) or HeLa P4 cell membranes (20). Briefly, membranes, ¹²⁵I-OS₂, and unlabeled sPLA₂s were incubated at room temperature in 0.5 mL of binding buffer (140 mM NaCl, 0.1 mM CaCl₂, 20 mM Tris-HCl, pH 7.4, and 0.1% bovine serum albumin). Incubations were started by addition of membranes and filtered after 1 h through GF/C glass fiber filters presoaked in 0.5% polyeth-

yleneimine and 10 mM Tris-HCl, pH 7.4. Iodination and binding experiments with the C-terminal chimera were performed as for WT OS₂. Cross-linking experiments on mouse brain membranes were performed as described (30) using 200 μM of suberic acid bis-*N*-hydroxysuccinimide ester (DSS, Sigma).

Central Neurotoxicity. Central neurotoxicity was evaluated by measuring the minimal lethal doses (LD₁₀₀) after intracerebroventricular injection of sPLA₂s (5 μL, dissolved in 0.9% NaCl) into BALB/c male mice (42–45 days old, 18–20 g) (58). LD₁₀₀ is defined as the lowest amount of sPLA₂ that kills all mice (*n* ≥ 2), half of this dose being not lethal 4 h after injection (*n* ≥ 2). All procedures were performed in accordance with the European Commission Council Directives.

Chick-Isolated Biventer Cervicis Muscle Assays. *Biventer cervicis* muscles were removed from male chicks (4–10 days-old); mounted in 5 mL organ baths; maintained at 34 °C under 1 g resting tension in Krebs solution of the following composition (mM): NaCl 118.4, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25.0, glucose 11.1, CaCl₂ 2.5; and bubbled continuously with 95% O₂ and 5% CO₂. Isometric contractions were measured via a Grass transducer (FT03) connected to a MacLab/8 system. Twitches were evoked by stimulating the motor nerve (supramaximal voltage, 0.2 ms, 0.1 Hz) via silver electrodes connected to a Grass S88 stimulator. Nerve-mediated (indirectly evoked) twitches were confirmed by abolition of twitches after addition of 10 μM (+)-tubocurarine (Sigma). In the absence of electrical stimulation, responses (directly evoked) to exogenous acetylcholine (ACh, Sigma, 1 mM, 30s), carbachol (CCh, 20 mM, 60 s), and potassium chloride (KCl, 40 mM, 30 s) were obtained prior to the addition of sPLA₂ and at the end of the experiment. This allows the classification of the sPLA₂ effects observed in the chick preparation into presynaptic or postsynaptic actions. Preparations were allowed to equilibrate for at least 30 min with continuous stimulation before addition of sPLA₂.

Myotoxic Effects on Mice. sPLA₂s were dissolved in phosphate-buffered saline (PBS, 0.12 M NaCl and 0.04 M sodium phosphate, pH 7.2). Aliquots of 100 μL, containing 5 μg of sPLA₂, were injected intramuscularly in the right thigh of CD-1 mice (18–20 g). Control mice received 100 μL of PBS under otherwise identical conditions. Three hours after injection, a blood sample obtained by cutting the tip of the tail was collected into heparinized capillary tubes and centrifuged. Plasma creatine kinase activity was determined using the Sigma kit No. 47-UV (Sigma-Aldrich). Creatine kinase activity is expressed in units U/L, where one unit is defined as the activity of the enzyme that produces 1 μmol of NADH/min under the conditions of the assay. Mice were observed for 8 h for symptoms of systemic toxicity. Statistical analysis was performed by determining the significance of the differences of the means of all experimental groups by ANOVA. Comparisons between pairs of means were performed by the Tukey Kramer method.

Anti-HIV-1 Activity. Single rounds of HIV-1_{BRU} virus infection in HeLa P4 cells were performed as described previously (20). Briefly, HeLa P4 cells were seeded into 24-well plates (80 000 cells per well) and infected the next day with 100 μL of HIV-1_{BRU} viral supernatant (100 ng of Gag p24). Virus and cells were left in contact for 8 h at 37 °C in

the absence or presence of the different sPLA₂s, and the culture medium was then replaced with fresh medium containing 50 μ M 3'-azido-3'-deoxythymidine (AZT, Sigma) to prevent another round of infection. Two days after infection, HeLa P4 cells were lysed, and β -galactosidase activity was measured as an index of virus infection.

Antimalarial Activity. Inhibitory effect toward *P. falciparum* intraerythrocytic development was assayed as previously described (21) in complete medium containing either 8% heat-inactivated human serum or 0.5% AlbuMAX II, a culture medium supplement based on calf serum lipid-enriched albumin (InVivoGen). Various concentrations of each sPLA₂ (100 μ L/well) were distributed in a 96-well plate, and then 100 μ L of a *P. falciparum* culture (1.5% parasitemia and 4% hematocrit) was added. After 24 h of incubation, 0.5 μ Ci ³H-hypoxanthine (ICN Biomedicals) was added per well, and parasites were grown for an additional 24 h. Plates were freeze-thawed and harvested on filters. Dried filters were moistened in scintillation liquid mixture (OptiScint, Hisafe) and counted in a 1450 Microbeta counter (Wallac, Perkin-Elmer).

RESULTS

Rationale for the Design and Recombinant Production of Wild-Type OS₂, Single Point Mutants, and Chimeras. Because the production of recombinant sPLA₂s can be problematic (11), we initially tried to express WT OS₂ from a synthetic gene in *Drosophila* S2 cells and *E. coli* using the pAB₃ expression vector system (see Experimental Procedures). Only the expression in *E. coli* was successful. Two different site-directed mutagenesis approaches were then developed to investigate the structure–function relationships of OS₂. First, we prepared single point mutants of OS₂ (glycine-30 to serine, histidine-48 to glutamine, and aspartate-49 to lysine) with the aim to abolish its enzymatic activity, but not its biological effects and/or binding properties to receptors (Figures 1 and 2). These three residues are absolutely conserved in all active sPLA₂s, are located in the active site or Ca²⁺-binding loop, and are known to be essential to catalysis (51). Moreover, venom sPLA₂s that harbor similar mutations occur naturally and are catalytically inactive, yet they can exert diverse pharmacological effects (59–61). Mutation of histidine-48 to glutamine has been shown to dramatically reduce enzymatic activity (62, 63). Mutations of glycine-30 and aspartate-49 are known to affect the binding of substrate and Ca²⁺, an essential cofactor for sPLA₂ activity and substrate binding (60, 64, 65). All three mutations were thus expected to produce catalytically inactive mutants of OS₂ for distinct reasons. We also constructed the OS₂ double mutant K31L–R34S to analyze the role of amino acids at positions 31 and 34, which are hypervariable within the highly conserved Ca²⁺-binding loop of sPLA₂s (66). Lysine-31 and arginine-34 of OS₂ were, respectively, replaced by leucine and serine which are found in the nontoxic porcine pancreatic sPLA₂ (52).

In the second approach, we designed chimeras between OS₂ and OS₁ in an effort to identify the main regions of OS₂ which are responsible for its molecular properties and biological effects. OS₁ was also purified from Taipan snake venom and is structurally homologous to OS₂, but has quite distinct biological properties (30, 52, 58). Pharmacologically, OS₁ binds only very weakly to N-type receptors, has a lower

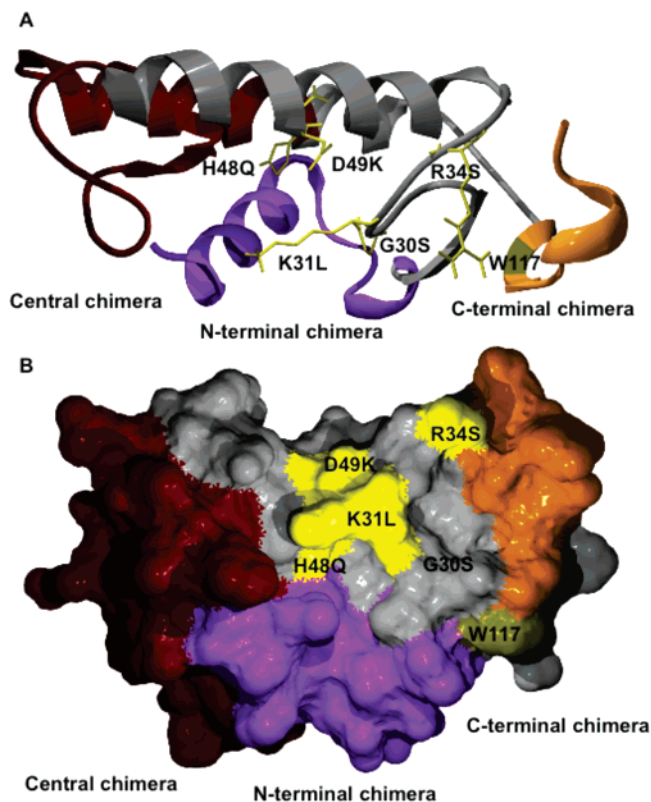


FIGURE 2: Structural model of WT OS₂ showing the location of the different mutations. An OS₂ model was built using the SwissPDB protein viewer software and the crystal structure of notexin (PDB 1AE7) as template. The location of the single point mutations and W117 are shown. The N-terminal, central, and C-terminal regions exchanged in the chimeras are shown in purple, brown-red, and orange, respectively. The conserved backbone between OS₁ and OS₂ is shown in gray. (A) Ribbon view of OS₂ with the putative interfacial binding surface facing down. (B) Molecular surface view after rotating the molecule by 90°.

specific activity on phospholipid substrates (see below), and is neither neurotoxic, myotoxic, nor able to potently inhibit HIV-1 replication ((20, 30) and below). However, both OS₁ and OS₂ are high-affinity ligands for the M-type receptor (58), and both also have antimalarial activity (see below). Structurally, OS₁ is 54% identical to OS₂ (Figure 1B). The level of identity is very high in the active site and Ca²⁺-binding loop, while it is much lower in the N-terminal, central, and C-terminal regions (Figure 1B). A three-dimensional model of OS₂ was produced by homology modeling using the X-ray structure of notexin as a template (Figure 2). A model for OS₁ (not shown) was also produced using as template the three-dimensional structure of the acidic sPLA₂ from *Ophiophagus hannah* venom (pdb structure 1gp7). Based on the major structural differences between the two proteins, we constructed three different chimeras (Figures 1 and 2). Importantly, all the residues in the protein segments that were exchanged in the chimera constructs and that point toward the core of the protein are identical between OS₁ and OS₂ except for the conservative change of Met-8 in OS₂ being replaced with Leu in OS₁. Thus, all but one of the residue changes occur at the molecular surface, and segment exchanges should not cause an adverse structural alteration of the chimeric proteins.

Recombinant Expression and Structural Characterization of OS₂, Single Point Mutants, and Chimeras in *E. coli*. All

Table 1: Recombinant Production of OS₂ and Its Mutants^a

sPLA ₂	yield of production (mg/L)	calculated mass (Da)	measured mass (Da)	Δ mass
OS ₂ WT	5	13317.08	13317.86	0.78
OS ₂ H48Q	2.6	13308.07	13307.27	0.80
OS ₂ D49K	6.4	13330.17	13329.84	0.33
OS ₂ G30S	1.3	13347.11	13347.89	0.78
OS ₂ K31L–R34S	1.5	13232.96	13233.79	0.83
N-terminal chimera	0.3	13278.02	13277.30	0.72
Central chimera	1	13992.66	13993.29	0.63
C-terminal chimera	2.6	13429.10	13430.12	1.02

^a All proteins were produced using the pAB3 *E. coli* expression system. The yield of production is the amount of pure protein obtained per liter of *E. coli* culture. The proteins were analyzed by MALDI-TOF mass spectrometry to confirm that all the disulfide bonds are formed and that proteins have not been covalently modified during purification.

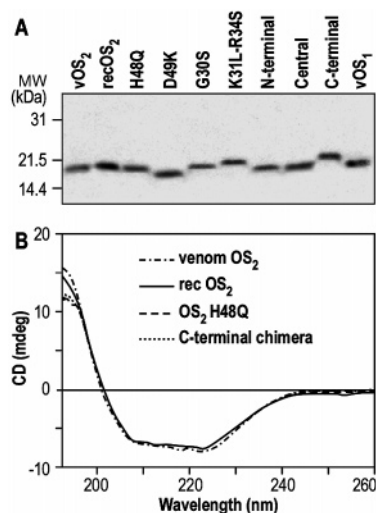


FIGURE 3: Analysis of the OS₂ mutants and chimeras by SDS–PAGE electrophoresis and CD. (A) One microgram of sPLA₂ was loaded on a 14% SDS–polyacrylamide gel under reducing conditions and stained with Coomassie brilliant blue. The apparent mobility appeared to be very sensitive to the pI of the protein. (B) CD spectra of recombinant WT OS₂, H48Q mutant, and C-terminal chimera compared to that of venom OS₂. The CD spectra of all the other mutants are identical to those shown here.

OS₂ proteins could be produced from *E. coli* with high yields (Table 1). The HPLC-purified proteins migrated as single bands of the expected size on a Laemmli SDS gel (Figure 3A). For all sPLA₂s, the molecular masses measured by MALDI-TOF mass spectrometry matched the calculated masses within less than 1 Da (Table 1), clearly establishing that all the disulfide bonds were formed in the proteins and that residue side chains had not been chemically oxidized or further modified during the refolding and purification processes. We also compared the overall conformation and secondary structure of venom, WT recombinant OS₂, and its mutants by CD spectroscopy. The CD spectra were very similar for all proteins, including the three chimeras (Figure 3B and not shown). Taken together, these results indicate that the recombinant proteins are pure and properly folded with all their disulfide bonds appropriately formed.

Lethal Potency of OS₂ and Its Mutants in Mice. Both venom and recombinant WT OS₂ are highly neurotoxic to mice following intracerebroventricular injection with an identical LD₁₀₀ of 2 μg/kg (Table 2). Interestingly, the active

Table 2: Biological Effects of OS₂ and Its Mutants^a

sPLA ₂ s	LD ₁₀₀	IC ₅₀ value (nM)			
		lethality (μg/kg)	HIV inhibition	Antimalarial activity	
				human serum	AlbuMAX II
venom OS ₂	2	35	3.1	>2500	
rec OS ₂ WT	2	33	2.1	>1250	
OS ₂ H48Q	33	>1000	>250	>1250	
OS ₂ D49K	>3330	>1000	>250	>1250	
OS ₂ G30S	3330	>1000	>250	>1250	
OS ₂ K31L–R34S	7	23	0.1	378	
N-terminal	>1500	>1000	>250	>1250	
Central	70	110	0.1	680	
C-terminal	33	60	17.9	>1250	
venom OS ₁	3500	300	0.07	1000	

^a Minimal lethal dose (LD₁₀₀) was measured by intracisternal injection of sPLA₂ into adult Balb/C male mice. LD₁₀₀ is defined as the lowest amount of sPLA₂ that is lethal to all mice (n ≥ 2), half of this dose being not lethal to mice 4 h after injection (n ≥ 2). HIV-1 inhibition was determined by infection of HeLa P4 cells with HIV-1_{BRU} in the absence or presence of various concentrations of sPLA₂ for 8 h. The level of viral infection was evaluated 2 days after infection. Antimalarial activity was measured by incubating various concentrations of sPLA₂ with an asynchronous culture of *P. falciparum* (1.5% parasitemia and 2% hematocrit) grown in the presence of 8% human serum or 0.5% AlbuMAX II. The inhibition of growth was determined by ³H-hypoxanthine incorporation. The IC₅₀ value is defined as the concentration of sPLA₂ that inhibits 50% of HIV-1_{BRU} infection or parasite growth. The values are representative of at least two independent experiments with SEM values lower than 50%.

site mutant H48Q is still neurotoxic (33 μg/kg), although the LD₁₀₀ is 16-fold lower than that of WT OS₂. Conversely, the two other active site mutants G30S and D49K have very low or no toxicity with LD₁₀₀ values of 3330 μg/kg or higher (Table 2). The double mutant K31L–R34S appears to be nearly as toxic as WT OS₂, suggesting that positions 31 and 34 are not critical, at least when mutated into leucine and serine. In marked contrast with WT OS₂, OS₁ displays a very low toxicity with an LD₁₀₀ of 3500 μg/kg. The N-terminal chimera is not toxic at a concentration as high as 1500 μg/kg and, thus, behaves similarly to OS₁. However, the central and C-terminal chimeras retain high toxicities, with LD₁₀₀ values of 70 and 33 μg/kg, respectively.

Enzymatic Properties of OS₂ and Its Mutants. The enzymatic properties of WT recombinant OS₂ were found to be identical to those of OS₂ purified from venom (Table 3). WT OS₂ shows a high-specific activity on *E. coli* membranes and is more efficient on the anionic phospholipid POPG than on the anionic phospholipid POPS or the zwitterionic phospholipid POPC (Table 3). As expected, mutations of G30, H48, and D49 in the Ca²⁺-loop and active site of OS₂ dramatically affected the catalytic activity, but did not alter the headgroup substrate specificity. Indeed, the specific activities of H48Q and G30S mutants were both reduced by about 500-fold. The activity of OS₂ D49K was reduced to undetectable levels. The mutations K31L and R34S in the Ca²⁺-loop did not affect the specific activity and only slightly modified the headgroup substrate specificity of OS₂.

In the case of the OS₂/OS₁ chimeras, a strong decrease in specific activity was observed for the N-terminal (1000-fold) and C-terminal (500-fold) chimeras (Table 3). Conversely, the central chimera retains a high-specific activity, which can be up to 5-fold higher than that of WT OS₂. Some changes in the headgroup substrate specificities were ob-

Table 3: Enzymatic Properties of OS₂ and Its Mutants^a

sPLA ₂	Specific activity				Interfacial binding
	<i>E. coli</i> (dpm/(pmol.min))	POPG (μ mol/(min.mg))	POPS (μ mol/(min.mg))	POPC (μ mol/(min.mg))	DOetPS/PC <i>K_d</i> (mM) ^d
venom OS ₂	1090	433	26	53.7	0.023
rec OS ₂ WT	1000	471	23	59.1	0.074
OS ₂ H48Q	2	1.2	0.05	0.05	0.040
OS ₂ D49K	0 ^b	nd ^c	nd ^c	nd ^c	no binding at 1 mM
OS ₂ G30S	2.3	0.60	0.03	0.03	no binding at 1 mM
OS ₂ K31L–R34S	950	169	3.10	61.8	0.007
N-term. chimera	1	2.60	0.003	0.02	no binding at 1 mM
Central chimera	5060	131	5.8	86	0.015
C-term. chimera	1.9	0.01	0.17	0.18	no binding at 1 mM
venom OS ₁	10	2.40	2.2	9.7	no binding at 1 mM

^a The specific activities were measured on [³H]oleate-radiolabeled *E. coli* membranes and on vesicles composed of the single phospholipid POPG, POPS, or POPC using the real-time fluorometric assay employing the fatty acid binding protein. Interfacial binding of sPLA₂ was determined by the centrifugation method on sucrose-loaded vesicles of 20 mol % DOetPS/80 mol % DOetPC. The results are representative of at least duplicate experiments with SEM values lower than 40%. ^b No sPLA₂ activity is detected at 1 μ M after 2 h of incubation. ^c Not determined. ^d The indicated *K_d* values are averaged values of *K_d* values measured by SDS–PAGE analysis (for sPLA₂s with low-specific activity), or by both SDS–PAGE and sPLA₂ assays (for sPLA₂s with high-specific activity).

served for the central chimera and the C-terminal chimera, which appeared to have a substrate specificity more similar to that of OS₁.

We also measured the binding properties of the different sPLA₂s to diether phospholipid vesicles made up of 20 mol % DOetPS/80 mol % DOetPC. WT OS₂ binds much more tightly to these phospholipid vesicles than OS₁ (Table 3). The double mutant K31L/R34S and the active site mutant H48Q also bind tightly, while G30S and D49K do not bind at all under our binding assay conditions. Among the three chimeras, the central chimera binds as tightly as WT OS₂, while the two other chimeras do not bind. When the binding of WT OS₂ was measured in the absence of free Ca²⁺ (2 mM EDTA instead of 2 mM CaCl₂ in binding buffer, see Experimental Procedures), the sPLA₂ was completely unable to bind to vesicles (not shown).

Binding of OS₂ and Its Mutants to M-Type and N-Type Receptors Measured by Competition Binding Assays with Iodinated OS₂. Binding studies on mouse and rabbit M-type receptor show that most mutants bind to this receptor with *K_{0.5}* values similar to that of WT OS₂ (Figure 4A and Table 4). The only exceptions were the N-terminal and central chimeras which show 4–30-fold lower affinities. The fact that all mutants including chimeras bind to the M-type receptor with high affinities further indicates that all of them are properly folded. Finally, the double mutant K31L/R34S was constructed to evaluate the contribution of residues 31 and 34 to M-type receptor binding. Indeed, we previously found that mutation of Leu-31 in the pancreatic sPLA₂ decreased its affinity for the receptor, suggesting that this position is important (52). We also noticed that the positions 31 and 34 are the only two hypervariable positions within the conserved Ca²⁺-loop, suggesting that the nature of the residues at these positions may determine, at least in part, the different binding properties of sPLA₂s to the receptor (52). In the OS₂ double mutant K31L/R34S, the OS₂ residues have been replaced by those of the pancreatic sPLA₂ with the goal to decrease the binding affinity. The fact that no decrease was observed suggests that the positions 31 and 34 are not crucial for the OS₂ interaction, or that these mutations can be accommodated in the OS₂ structure to maintain a high affinity. Further work including cocrystal-

lization of the sPLA₂–receptor complex is required to address the contribution of residues at positions 31 and 34 in the sPLA₂–M-type receptor interaction.

We next investigated the binding properties of the different mutants to N-type receptors present in mouse brain membranes and human HeLa P4 cells. These two sources of N-type receptors were chosen because of their potential implication in two of the pharmacological models used in this study, that is, neurotoxicity in mouse brain and inhibition of HIV replication. Binding to N-type receptors was found to be sensitive to the different mutations analyzed, but to a different extent (Figure 4B,C and Table 4). The mutant K31L–R34S retained an affinity close to that of WT OS₂ for both mouse brain and HeLa P4 N-type receptors. The mutant H48Q was also able to efficiently bind to the N-type receptors expressed in mouse brain and HeLa P4 cells, yet its affinity was 50-fold lower than that of WT OS₂. D49K and the C-terminal chimera also bind to the N-type receptors with nanomolar affinities, even though their affinities are 180–1230-fold lower than that for WT OS₂. Mutation of glycine-30 produces a very dramatic effect, decreasing the affinity by a factor of about 20 000. Finally, the N-terminal and central chimeras were unable to compete with iodinated OS₂ for binding to mouse brain and HeLa P4 cell membranes.

Binding of OS₂ and Its Mutants As Revealed by Cross-Linking of Labeled OS₂ to Mouse Brain Membranes. Because some of the above-mentioned binding results did not correlate well with the central neurotoxic properties of OS₂ mutants (Table 2), while a relationship between sPLA₂ neurotoxicity and N-type receptor binding was previously proposed (30, 67), we analyzed the binding properties of the OS₂ mutants by cross-linking experiments of radiolabeled OS₂ to mouse brain membranes (Figure 5). These experiments constituted another method to visualize the ability of the OS₂ mutants to bind to N-type receptor proteins. When ¹²⁵I-OS₂ was allowed to bind to mouse brain membranes and subsequently cross-linked with DSS, several proteins of 14, 18, 39, and 50 kDa were labeled (Figure 5). The cross-linking of these proteins is specific because it is fully prevented by an excess of unlabeled OS₂ (300 nM), but not OS₁, which is unable to bind to N-type receptors (Table 4). In agreement with the competition binding assays (Table 4), the mutant K31L–

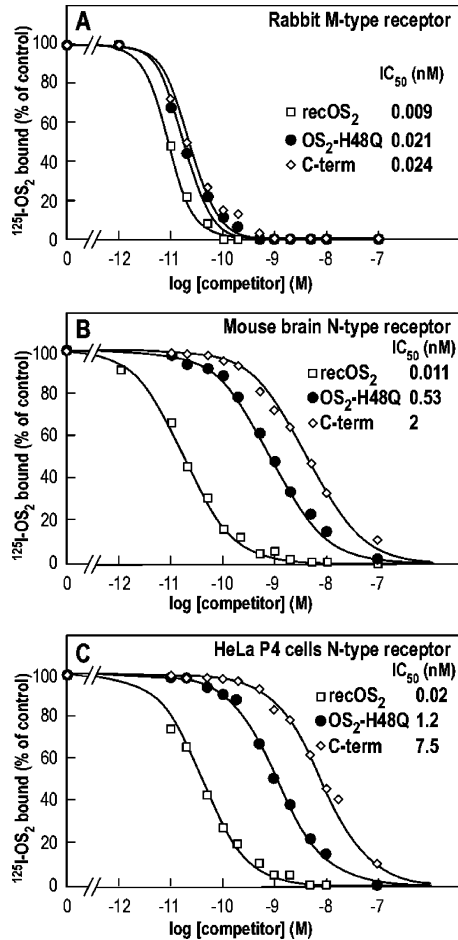


FIGURE 4: Binding properties of OS₂ and its mutants to M- and N-type receptors measured by competition assays with iodinated WT OS₂. Competition experiments between ¹²⁵I-OS₂ and unlabeled WT recombinant OS₂ (□), the mutant H48Q (●), and the C-terminal chimera (◇) for binding to (A) the cloned rabbit M-type receptor expressed in HEK 293 cells, (B) N-type receptors from mouse brain membranes, and (C) N-type-like receptors from HeLa P4 cell membranes. Membranes were incubated with ¹²⁵I-OS₂ and various concentrations of unlabeled competitors. Results are expressed as percentage of the ¹²⁵I-OS₂ specific binding measured in the absence of competitor. The membrane concentration was adjusted to obtain a specific binding of 10–20% of the total radiolabeled ligand added (55 pM). The nonspecific binding was measured in the presence of 100 nM unlabeled OS₂ and was below 10% of total binding.

R34S completely prevented the cross-linking of ¹²⁵I-OS₂ to the N-type receptors. Similarly, the mutants H48Q and D49K decreased the labeling, whereas the mutant G30S and the N-terminal chimera had no effect. However, the central and C-terminal chimeras appeared more potent than expected from the competition assays (Figure 4), especially for the C-terminal chimera which efficiently prevented the cross-linking of iodinated OS₂.

Binding Properties of Iodinated C-Terminal Chimera. To reconcile the above results, we postulated that WT OS₂ binds with high affinities to at least two different populations of binding sites in mouse brain, while the central and C-terminal chimeras bind with high affinity to only a subset of these receptors. This receptor subset would correspond to those visualized by cross-linking experiments (Figure 5). According to this hypothesis, the WT OS₂-specific population(s) of binding sites (which could be proteins and/or phospholipids) should be abundant in mouse brain membranes and

Table 4: Binding Properties of OS₂ and Its Mutants to M-Type and N-Type Receptors^a

sPLA ₂ s	K _{0.5} value			
	rabbit M-type (nM)	mouse M-type (nM)	mouse N-type (nM)	HeLaP4 N-type (nM)
venom OS ₂	0.009	0.07	0.011	0.02
rec OS ₂ WT	0.009	0.07	0.011	0.02
OS ₂ H48Q	0.021	0.19	0.53	1.2
OS ₂ D49K	0.026	0.49	13.5	0.96
OS ₂ G30S	0.035	0.2	200	400
OS ₂ K31L–R34S	0.008	0.047	0.045	0.028
N-terminal chimera	0.19	1.4	>500	>500
Central chimera	0.037	2.2	>300	>300
C-terminal chimera	0.024	0.03	2	7.5
venom OS ₁	0.1	0.15	>1000	>1000

^a Competition binding assays between iodinated OS₂ and unlabeled mutants or chimeras were performed on recombinant M-type receptor or N-type receptors from mouse brain membranes and HeLa P4 cell lysates. The K_{0.5} value is defined as the concentration of sPLA₂ competitor which inhibits 50% of the specific binding. K_{0.5} values are representative of at least three independent experiments with SEM values lower than 30%.

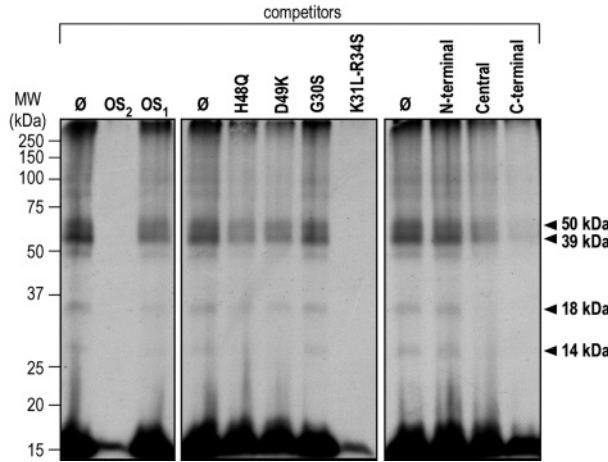


FIGURE 5: Binding properties of WT OS₂ and its mutants as revealed by cross-linking experiments of WT ¹²⁵I-OS₂ to mouse brain membranes. Membranes (300 μg of protein/mL) were incubated with 200 pM ¹²⁵I-OS₂ in the absence (Ø) or presence of unlabeled sPLA₂s (300 nM), centrifuged, cross-linked with 200 μM DSS, and loaded on a 10% SDS–polyacrylamide gel under reducing conditions. The gel was stained with Coomassie brilliant blue, dried, and autoradiographed at –70 °C for 7 days. After correction for the molecular mass of labeled OS₂ assuming a 1:1 stoichiometry, OS₂ binding proteins with molecular masses of 14, 18, 39, and 50 kDa are detected. The most representative experiment out of three experiments performed on two different mouse brain membranes is shown.

responsible for most of the signal measured with iodinated WT OS₂ in competition binding assays (Figure 4). Additionally, the two chimeras should have weak affinity for these binding sites, and thus, they should not compete efficiently (Figure 4).

To investigate this hypothesis, we iodinated the central and C-terminal chimeras to perform direct binding studies and identify a subset of OS₂ binding sites. Binding assays with the central chimera could not be done as it became highly oxidized during the iodination procedure with lactoperoxidase (data not shown), even when iodination was performed under very mild conditions (68). Conversely, the C-terminal chimera could be iodinated without oxidation and

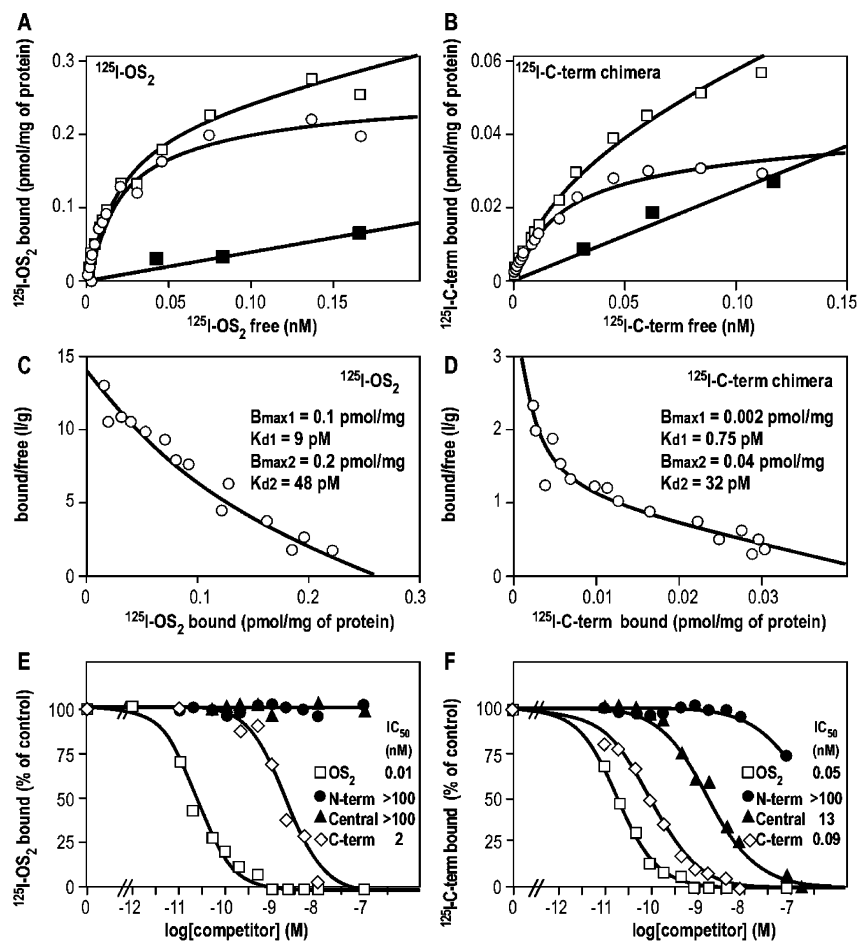


FIGURE 6: Comparison of the binding properties of iodinated WT OS₂ and C-terminal chimera to mouse brain membranes. All of the binding experiments were performed in parallel and on the same membrane preparations ($n = 3$). (A and B) Direct equilibrium binding assays with the two iodinated ligands. Membranes were incubated with various concentrations of labeled ligands in the absence (□) or presence (■) of 100 nM unlabeled homologous ligand. The specific binding (○) represents the difference between total (□) and nonspecific binding (■). (C and D) Scatchard plot analysis of the specific binding. (E and F) Competition experiments between iodinated ligands and unlabeled WT recombinant OS₂ and the different chimeras. Membranes were incubated with 50 pM of WT ¹²⁵I-OS₂ or ¹²⁵I-C-terminal chimera and various concentrations of unlabeled competitors. Results are expressed as the percentage of specific binding measured in the absence of competitor. The nonspecific binding was measured in the presence of 100 nM unlabeled homologous ligand and was below 15% of total binding.

used in direct binding experiments. Figure 6 shows a comparison of the binding properties of iodinated WT OS₂ and C-terminal chimera obtained in parallel experiments on the same mouse brain membrane preparations. Figure 6 (panels A and B) shows that the binding of both proteins to mouse brain membranes is saturable and specific. In both cases, the Scatchard plots of the specific binding are best fitted by curvilinear curves, suggesting that the two ligands bind to at least two distinct families of binding sites with very high affinities in the picomolar range (Figure 6C,D). However, the total number of binding sites for the chimera (0.042 pmol/mg of total protein) appears to be about 7-fold lower than that for WT OS₂ (0.3 pmol/mg of total protein). This result and the fact that WT OS₂ competes with the labeled chimera for binding (Figure 6F) indicate that the chimera indeed binds to a minor subset of WT OS₂ binding sites. Additionally, when the competition binding assays for the different chimeras were performed using the iodinated C-terminal chimera as labeled ligand, the affinities were much higher than those obtained using labeled WT OS₂ (Figure 6E,F). Together, the binding properties of the labeled C-terminal chimera support the hypothesis of at least two major populations of OS₂ binding sites, among which one

binds with high affinity the central and C-terminal chimeras and has molecular masses corresponding to those revealed by the cross-linking experiments (Figure 5). The curvilinear curve obtained with the iodinated C-terminal chimera suggests that the binding sites recognized by WT OS₂ and the C-terminal chimera are still complex in nature and may be represented by several independent targets.

Effects of OS₂ and Its Mutants on Neuromuscular Transmission. Neurotoxic snake venom sPLA₂s are called β -neurotoxins because they affect neuronal or neuromuscular transmission by acting presynaptically, even though some of them can also exert postsynaptic and myotoxic effects (2, 14, 69). OS₂ is lethal when injected intracerebroventricularly into rat or mouse brain (30, 48, 70) and has been shown to block acetylcholine release in a neuro-neuronal synapse in *Aplysia* (49), suggesting that OS₂ is also a β -neurotoxin. To confirm that OS₂ acts presynaptically at a vertebrate neuromuscular junction, we analyzed its effects and those of its mutants on the chick *biventer cervicis* neuromuscular preparation (Figure 7). Only WT OS₂ and the K31L-R34S mutant, when tested at 200 nM, were able to reduce the height of indirect twitches evoked by electrical nerve stimulation (Figure 7A). At lower concentrations of 20 and

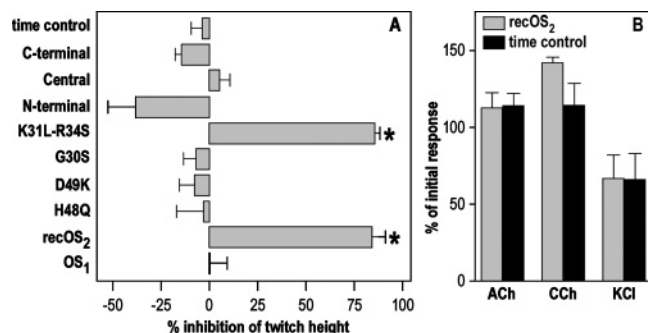


FIGURE 7: Effects of OS₂ and its mutants on neuromuscular transmission in the chick *biventer cervicis* muscle preparation. (A) Effects of recombinant OS₂, OS₁, mutants, and chimeras (200 nM) on indirect twitches evoked by electrical nerve stimulation. The percentage of inhibition of twitch height was measured 3 h after the addition of sPLA₂ to the bath. (B) Effects of WT recombinant OS₂ on contractions of the chick *biventer cervicis* preparation in response to acetylcholine (ACh, 1 mM), carbachol (CCh, 20mM), and potassium chloride (KCl, 40 mM) compared to time control (black bar). All experiments were performed in triplicate. (*) $p < 0.001$, significantly different from time control (unpaired t -test assuming unequal variance).

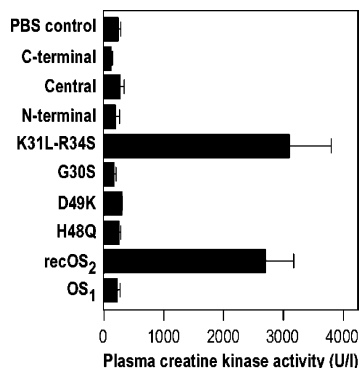


FIGURE 8: Myotoxic effects of OS₂ and its mutants. Three hours after intramuscular injection of sPLA₂ (5 μ g) into mice, the plasma was collected, and creatine kinase activity was measured as an index of myotoxicity. One unit of creatine kinase activity is defined as the amount of enzyme that produces 1 μ mol of NADH/min.

50 nM, WT OS₂ had no twitch reduction effect, indicating that OS₂ is not very potent on this neuromuscular preparation. In line with this rather low potency, all the other mutants and chimeras were found to be inactive when assayed at 200 nM. We next analyzed the effect of WT OS₂ on contractions of the chick *biventer cervicis* preparation directly evoked by various agonists including acetylcholine, carbachol, and potassium chloride (Figure 7B). No significant inhibition of the contractions was observed in the presence of WT OS₂ (Figure 7B) or mutants (not shown) at 200 nM.

Myotoxic Effects of OS₂ and Its Mutants. We also analyzed whether OS₂ can exert myotoxic effects when injected intramuscularly into mice (Figure 8). Only the groups of mice injected with recombinant WT OS₂ and the mutant K31L–R34S showed signs of myotoxic intoxication, as measured by plasma creatine kinase activity. In both cases, mice also developed signs of neurotoxicity, with paralysis of the limbs and respiratory difficulties. Three out of the five mice injected with recombinant WT OS₂ and the mutant K31L–R34S died within 8 h. In addition, mice in these two experimental groups showed macroscopic swelling and immobilization of the injected limb consistent with myotoxic effects, as previously observed for other myotoxins (71). In

contrast, mice injected with the other mutants, chimeras, and venom OS₁ did not show any evidence of intoxication nor local myotoxic effects at the site of injection.

Anti-Plasmodium Activity of OS₂ and Its Mutants. WT OS₂ and its mutants were tested for their ability to inhibit the intra-erythrocytic growth of *P. falciparum*, the causative agent of the most severe form of human malaria (Table 2). The effect of sPLA₂s was assayed in the presence of human serum (8%) or in a semi-defined medium (0.5% AlbuMAX II, a serum substitute devoid of lipoproteins) to evaluate whether sPLA₂s act directly or indirectly by modification of serum components including hydrolysis of lipoprotein phospholipids (72). In the presence of serum, both OS₁ and WT OS₂ inhibited *P. falciparum* growth. Remarkably, OS₁ was even more potent than OS₂ (Table 2). The mutants H48Q, D49K, G30S, and the N-terminal chimera, that display extremely low enzymatic activities on all types of phospholipids (Table 3), were totally inactive against *P. falciparum*. Conversely, the mutant K31L/R34S, the central chimera, and to a lesser extent the C-terminal chimera were still able to inhibit the growth of the parasite. The results were dramatically different in the presence of AlbuMAX, where most of the sPLA₂s were inactive. Under those conditions, only the sPLA₂s which are extremely potent in the presence of human serum were still capable of preventing the growth of the parasite, yet at much higher concentrations (Table 2).

Inhibition of HIV Replication by OS₂ and Its Mutants. We previously found that several but not all venom sPLA₂s can block the entry of HIV-1 into host cells (20). Venom and recombinant WT OS₂ as well as the mutant K31L–R34S were found to be inhibitors of HIV-1 infectivity with IC₅₀ values in the nanomolar range (Table 2). Conversely, at concentrations up to 1 μ M, the mutants H48Q, D49K, G30S, and the N-terminal chimera did not inhibit HIV-1 infection. However, the central and C-terminal chimeras still retained a fairly potent antiviral activity, with IC₅₀ values between those of WT OS₂ and OS₁ (Table 2).

DISCUSSION

A large number of studies using in silico protein sequence comparisons, chemical modification of amino acids, and more recently site-directed mutagenesis on different toxic sPLA₂s have been carried out over the past 30 years (23, 35, 43). However, the results from these studies have remained difficult to interpret for several reasons: (i) sPLA₂s are quite compact molecules, and some mutations may cause structural changes at sites remote from the mutation site; (ii) they probably exert most of their biological properties through relatively large molecular surfaces encompassing several discrete domains (i.e., the N-terminal helix, Ca²⁺-loop, etc.); (iii) their mechanism of action may include several steps (39, 40); (iv) several studies have been based on chemical modification or mutagenesis of single amino acids, which in most cases have led to only relatively modest effects; (v) the results obtained for a particular venom sPLA₂ may not apply to other sPLA₂s; and (vi) some amino acids that are important for catalytic activity may also be involved in receptor binding or biological activities.

The main goal of our study was to obtain new insights into the structure–function relationships of venom sPLA₂s using OS₂ from the Australian Taipan snake venom as a

template, and with a particular focus on its central neurotoxic effects. Our first approach employed site-directed mutagenesis at the active site which, to our knowledge, has never been used to establish the relationship between enzymatic catalysis and central neurotoxicity. The second approach used large chimeras between OS₁ and OS₂ and is based on the observation that the two proteins from the same Taipan venom display very distinct catalytic, toxic, and N-type receptor binding properties. The aim of this second approach was to produce very dramatic changes in the molecular and pharmacological properties of OS₂ and thus map unambiguously which of the three regions plays the major role. A similar chimeric approach was used for ammodytoxins, but in that case, the predicted C-terminal neurotoxic region of ammodytoxin A was introduced into the nontoxic ammodytoxin I2, and only mild effects were observed (34). As discussed below in more details, our results clearly indicate that: (i) based on the clear dissociation observed for the H48Q mutant and the C-terminal chimera, the catalytic activity of OS₂ is responsible for little, if any, of the central neurotoxicity; (ii) the N-terminal region of OS₂ and some well-conserved residues of the Ca²⁺-loop, but not the central and C-terminal regions, are key elements for central neurotoxicity and several other pharmacological effects; (iii) the same N-terminal region allows OS₂ to bind to a subset of N-type receptors which is likely to be associated with central neurotoxicity; and (iv) the antimalarial effect of OS₂ and OS₁ appears to be dependent on the catalytic activity of these enzymes on serum lipoproteins. Together, these findings support the view that a single sPLA₂ exerts its different pharmacological effects through distinct mechanisms that depend or not on its enzymatic activity (2).

Enzymatic Properties of the OS₂ Mutants and Chimeras. Our two approaches have generated OS₂ mutants which have dramatically lower enzymatic activity for different reasons. The single point mutants G30S, H48Q, and D49K have sPLA₂ activity reduced by 500-fold or more, essentially because they cannot efficiently bind or hydrolyze phospholipids in their active site (51). Conversely, the N-terminal and C-terminal chimeras have reduced activity because they cannot efficiently bind to the phospholipid membrane interface. Indeed, the sPLA₂ catalysis consists of two distinct steps, in which the enzyme first binds to a surface of about 40–50 phospholipids at the phospholipid–water interface (interfacial binding) and then forms a Michaelis–Menten complex by binding a single phospholipid molecule into the active site (51). These two steps involve two topologically distinct sPLA₂ domains, that is, the interfacial binding surface and the active site slot. The active site contains six residues which are conserved in all active sPLA₂s among which G30, H48, and D49 play major catalytic roles. Conversely, the interfacial binding surface comprises at least 10–15 residues which are not conserved between sPLA₂s and which can be located in the N-terminal, central, and C-terminal regions of sPLA₂s. The nature of these residues will determine the affinity of the enzyme for different lipid interfaces. Over the years, it has become clear that the interfacial binding of sPLA₂s is the key step that governs and explains most of the differences in specific activities on different phospholipid substrates. From a large number of studies, first performed on venom sPLA₂s and then on the various mammalian enzymes, it is now clear that while basic, neutral, and

aliphatic residues found in the interfacial binding domain contribute to phospholipid binding, a few aromatic residues, especially tryptophans, play major roles in sPLA₂ binding, particularly to zwitterionic phospholipids (73–81).

The 500–1000-fold drop in catalytic activity of the N-terminal and C-terminal chimeras is largely explained by their decreased binding affinity for phospholipid vesicles (Table 3). This indicates that the N-terminal and C-terminal regions of OS₂, but not the central one, contain key residues which are involved in high-affinity interfacial binding and are not present in OS₁. Among these residues, OS₂ has a tryptophan located in the C-terminal region (Figure 2 and position 117 in Figure 1) that would explain at least in part why the C-terminal chimera is less active than WT OS₂ (Table 3). Accordingly, mutation of asparagine to a tryptophan at this position (N117W) in the pancreatic group IB sPLA₂ results in a 18-fold increase in interfacial binding (78). OS₂ has a second tryptophan at position 75 (Figure 1), but this residue is located on the β -wing structure in the central region, far away from the interfacial binding domain and, thus, most probably does not participate in interfacial binding. This is consistent with the fact that, for many sPLA₂s, the critical interfacial binding residues are present in the N-terminal and C-terminal regions (79, 80, 82–86). The slightly altered enzymatic activity and substrate specificity of the central chimera (Table 3) may be related at least in part to the deletion of the OS₁ pancreatic loop, whose deletion in the pancreatic group IB sPLA₂ produced similar effects (87).

The H48Q mutant does not show altered interfacial binding under our experimental conditions, indicating that the drop in activity is essentially due to its inability to hydrolyze phospholipids in the active site. Conversely, the very low enzymatic activity of the OS₂ active site mutants G30S and D49K is due not only to their inability to bind the substrate at the active site, but also to altered interfacial binding (Table 3). Previous studies on the pancreatic group IB and human group IIA sPLA₂s have shown that the mutations G30S, H48Q, and D49K largely alter phospholipid catalysis, but have little effects on interfacial binding (62–65, 88). Similar conclusions were drawn from enzymatic studies and crystal structures of venom sPLA₂s harboring the natural mutations G30S, H48Q, and D49K (59–61, 89). However, detailed studies on the role of the Ca²⁺ cofactor in catalysis by pancreatic and bee venom sPLA₂s indicated that the interfacial binding and the binding at the active site are interconnected, where the Ca²⁺-dependent binding of the substrate at the active site partially drives the interfacial binding of sPLA₂ by mass action (81, 90, 91). Under our phospholipid vesicle assay conditions (20 mol % DOetPS/80 mol % DOetPC), the mutants G30S and D49K do not bind efficiently to the vesicles, which can be explained at least in part by the fact that they cannot bind Ca²⁺ and thus their substrate at the active site. This result also agrees with the absence of vesicle binding for WT OS₂ when Ca²⁺ is omitted (not shown). Additionally, the structural data accumulated so far also suggest that the mutations G30S, H48Q, and D49K locally alter to different extents the conformations of the Ca²⁺-loop and N-terminal helix, with conformational changes induced by the mutation H48Q being smaller than those induced by mutation of Asp-49 (61, 63, 92, 93). On the other hand, deletion of the C-terminal region of the pancreatic sPLA₂ affects substrate binding at the active site

(90). In the case of His-48 alkylation, an allosteric coupling between the catalytic site and the membrane binding surface of the sPLA₂ has been proposed (43–46, 94). Furthermore, the hydrogen bonding network connecting the active site to the interfacial binding domain and which involves His-48 is likely to be important for the conformational stability of sPLA₂s (46, 92). Finally, a recent study showed that binding of a hydrophobic ligand in the active site of a Lys-49 venom sPLA₂ induces conformational changes in the C-terminal domain (95). In summary, it is likely that the drop in activity observed in the case of the OS₂ active site mutants (Table 3) is due in large part to the point mutation affecting a key catalytic functionality, that is, phospholipid hydrolysis for the H48Q and Ca²⁺-dependent phospholipid binding at the active site for G30S and D49K. However, we propose that these mutations may also induce small (H48Q) or significant (G30S and D49K) conformational changes at the surface of the protein, especially on the N-terminal α -helix. These conformational changes would represent the molecular basis for the loss of central neurotoxicity and binding properties to N-type receptors of these mutants (see below).

Probing the Role of sPLA₂ Activity in Central Neurotoxicity of OS₂. Our first aim was to analyze the role of sPLA₂ activity by preparing catalytically inactive mutants of OS₂ and determining whether these mutants are still neurotoxic when injected intracerebroventricularly into mice. OS₂ is one of the most neurotoxic sPLA₂s by this route (30). The results obtained with the mutant H48Q indicate a clear dissociation, since this mutant is only 16-fold less neurotoxic, but 500-fold less catalytically active, than WT OS₂. This result is in accordance with the partial dissociation obtained in the past by alkylation of His-48 on several neurotoxic sPLA₂s (41–43, 96). The results obtained with the mutants G30S and D49K support the view that sPLA₂ activity may be required for neurotoxicity, since these two mutants have lost both properties (Tables 2 and 3). However, as explained above, their loss of neurotoxicity may be due to a conformational change at the surface of the protein induced by the point mutation. It may be also possible that surface residues from the Ca²⁺-loop including G30 more directly participate in the interaction with the neurotoxic cellular targets. Our data obtained with the central and C-terminal chimera further indicate that there is no direct correlation between enzymatic activity and neurotoxicity. Indeed, the central chimera is at least as active as WT OS₂, but has a 35-fold lower neurotoxicity, and the C-terminal chimera has 500-fold lower activity, but only 16-fold less central neurotoxicity. The fact that both the H48Q mutant and the C-terminal chimera have dramatically reduced enzymatic activity on both anionic and zwitterionic phospholipids likely indicates that their catalytic activity is also very low on cellular phospholipids *in vivo*, although this hypothesis should deserve attention in future studies.

The fact that the H48Q mutant and the C-terminal chimera retain most of the central neurotoxicity of WT OS₂, yet show a much more drastic loss in enzymatic activity on all types of anionic and zwitterionic phospholipid substrates (Table 3), indicates that the enzymatic activity is at most a minor factor involved in central neurotoxicity. If we assume that the 16-fold decrease in central neurotoxicity is due to mutations that cause a small structural change in the neurotoxic site of OS₂ which in turn affects the energetics

of its binding to brain components required for central neurotoxicity (a 16-fold factor would correspond to a loss of binding energy to brain component(s) of about 1.5 kcal/mol), the catalytic activity of OS₂ would in fact not be required for central neurotoxicity. According to this hypothesis, the neurotoxic action of OS₂ would simply be due to the specific binding of the sPLA₂ to its cellular neuronal targets via the neurotoxic site. OS₂, and possibly other neurotoxic sPLA₂s, would then behave like many other distinct neurotoxins which are devoid of catalytic activity and bind to specific membrane targets (97–99). This view is reminiscent of the situation observed for the K-49 myotoxic sPLA₂s, which exert their myotoxic effect independently of enzymatic activity, but via a myotoxic site located on the C-terminal region of the sPLA₂ (36). It is also reminiscent of the fact that the major anticoagulant properties of sPLA₂s do not require enzymatic activity, but rather a specific binding to factor Xa (15, 100). The production of OS₂ mutants fully devoid of catalytic activity yet still highly neurotoxic is however required to validate this hypothesis.

Mapping the Region of OS₂ Essential for Its Central Neurotoxicity. The results obtained with the chimeras indicate that the N-terminal region of OS₂ contains key residues which are not present in OS₁ and constitute the major structural determinants of the neurotoxic site. The slight decrease in central neurotoxicity observed for the central and C-terminal chimeras suggests that a few residues from these regions contribute to neurotoxicity, or that their mutations mildly affect the structure of the N-terminal region. Similarly, the results obtained with the mutants G30S and D49K indicate that strictly conserved residues from the Ca²⁺-loop are either key structural elements of the neurotoxic site or that these residues contribute to the conformational stability of the neurotoxic site. The neurotoxic site of OS₂ is thus overlapping with the interfacial binding domain, and this explains why the sPLA₂ activity seems to be involved, although no obvious correlation can be found. The proposed location of the OS₂ neurotoxic site agrees with several other studies based on protein sequence alignments, crystal structure analyses, amino acid modifications, and site-directed mutagenesis (35, 43, 61, 67, 84, 96, 101). However, it is in apparent contradiction with some studies indicating that the β -wing or the C-terminal domains are involved ((47, 102, 103) and references therein). Importantly, we note that the modifications or mutations performed in these latter studies led in most cases to a small decrease in neurotoxicity, lower than 10-fold, which is comparable to the relatively modest 16- and 35-fold decreases observed here for the central and C-terminal chimeras. Our results using the single point mutations D49K, G30S, and the chimeras led to more consistent decrease or even complete loss of neurotoxicity, unambiguously indicating that the Ca²⁺-loop and the N-terminal region are crucially involved. Finally, it is remarkable that the single point mutation G30S completely inactivates OS₂ and virtually converts this latter into OS₁ in terms of molecular and functional properties (except for the antimalarial activity, see below). The same is also observed for the N-terminal chimera, and a similar result was previously observed for the bee venom sPLA₂ when mutated at the functionally homologous glycine-12 (67).

Binding Properties of the OS₂ Mutants and Chimeras to M-Type and N-Type Receptors and Relationship with Central Neurotoxicity. The pancreatic sPLA₂ has previously been shown to bind to the M-type receptor through residues located in or close to the Ca²⁺-loop, and this binding leads to an inhibition of sPLA₂ activity (25, 32). However, the binding does not appear to be related to enzymatic activity, since both catalytically active and inactive enzymes can bind to the M-type receptor (25, 32, 50). In the case of OS₂, we also found that the active site mutants can bind to rabbit or mouse M-type receptors with high affinity (Table 4). Since the active site residues, in particular His-48 and Asp-49, probably do not directly interact with residues from the receptor, it is likely that the small shifts in affinity observed for the H48Q and D49K mutants (Table 4) are due to mutation-induced conformational changes occurring on the molecular surface of OS₂ (see above). For chimeras, we found that they all bind to the M-type receptor. This result was expected, since both OS₁ and OS₂ bind to this receptor (58). Since OS₁ and both neurotoxic and non-neurotoxic mutants of OS₂ can bind with similar affinities to this receptor, while several other neurotoxic sPLA₂s do not bind to this receptor (58), it is unlikely that the M-type receptor plays a critical role in central neurotoxicity. This contrasts with the proposed role of the M-type receptor in the neurotoxicity of ammodytoxins (39).

The N-type receptors for OS₂ and other neurotoxic sPLA₂s were initially identified in rat brain membranes (30), and then in several peripheral tissues and cells (25), including the HeLa P4 cells used for HIV studies (see below). Although not yet cloned, these receptors are likely to form a family of binding proteins with molecular masses distinct from that of the M-type receptor (25). The physiological roles of these receptors are still unclear, but they have been proposed to participate in the neurotoxic and anti-HIV activities of venom sPLA₂s (20, 30). The binding properties of the different mutants to the N-type receptors expressed in mouse brain microsomal membranes and HeLa P4 cells follow the same trend. As measured by competition studies using iodinated WT OS₂, mutations in the active site decrease the affinity of OS₂ for the N-type receptors, but there is no obvious relationship between receptor affinity and enzymatic activity or interfacial binding to purified lipid vesicles. As discussed above, the shift in affinity may be explained by the fact that mutations in the active site lead to conformational changes in the Ca²⁺-loop and N-terminal surface of OS₂ that directly affect the binding properties. This explanation appears more likely than the one assuming that the receptor has a molecular arm penetrating directly into the active site. A similar shift in affinity was observed when OS₂ was alkylated by *p*-bromo-phenacyl-bromide (unpublished data). The absence of relationship between binding and enzymatic activity was also observed for the chimeras, where all three possibilities were found, that is, loss of both receptor binding and enzymatic activity (N-terminal chimera), loss of binding but not sPLA₂ activity (central chimera), or loss of more enzymatic activity than binding (C-terminal chimera).

The apparent discrepancies observed between cross-linking experiments and competition assays with the central and C-terminal chimeras against labeled WT OS₂ prompted us to investigate their direct binding properties. The radiolabeled C-terminal chimera appeared to bind to a minor subset of

OS₂ binding sites which displays high affinity for WT OS₂, as well as for the central and C-terminal chimeras, but not for the N-terminal chimera (Figure 6). These results restore at least partially the correlation between the neurotoxicity and the affinity of OS₂ mutants for this subset of N-type binding sites and, thus, suggest that the high-affinity binding sites for the C-terminal chimera are more directly involved in central neurotoxicity. The molecular nature and complexity of these binding sites are unknown, but they most likely consist of several proteins, as revealed by the cross-linking experiments and the curvilinear Scatchard plot analysis (Figures 5 and 6). The nature of the other subset of WT OS₂ binding sites on which the C-terminal chimera binds with low affinity is also unknown, but the fact that the C-terminal chimera cannot bind efficiently to phospholipid vesicles raises the possibility that they are lipidic in nature. These latter binding sites would not be important for central neurotoxicity. The N-terminal region of OS₂, but not the central region, appears to be critical for the interaction with the two subsets of N-type OS₂ binding sites. On the other hand, the C-terminal region of OS₂ appears important only for binding to the subset which may be lipidic in nature. The critical role of the N-terminal region in central neurotoxicity of OS₂ and interaction with these subsets of N-type receptors is in agreement with the results previously obtained for bee venom sPLA₂ (67) and other neurotoxic sPLA₂s including taipoxin, Pa-11, crotoxin (104), and ammodytoxins ((105) and references therein). The relationships between the ammodytoxin binding proteins which have been identified (calmodulin, R25, and 14-3-3 protein isoforms) and these subsets of N-type receptors are still unclear. However, our results are in agreement with some of the structure–function studies on ammodytoxins indicating that residues in the N-terminal and C-terminal regions, but not the β -wing (which is within the OS₁/OS₂ region swapped in the central chimera), are involved in binding to calmodulin and R25 (34, 47, 84).

Inhibition of Neuromuscular Transmission and Myotoxic Effects Induced by OS₂. Similarly to many neurotoxic snake venom sPLA₂s which are called β -neurotoxins and include β -bungarotoxin, crotoxin, textilotoxin, notexin, and taipoxin (69, 106), we found that OS₂, but not OS₁, acts presynaptically on a chick *biventer cervicis* neuromuscular preparation, indicating that OS₂ is also a β -neurotoxin. At 200 nM, we did not observe any evidence for a postsynaptic effect, which fits with the fact that β -neurotoxins exert postsynaptic effects only at concentrations higher than those required for their neurotoxic effects (107). Except for K31L/R34S, none of the OS₂ mutants had any inhibitory effect at this concentration on the chick *biventer cervicis* preparation, hampering the analysis of the structure–function relationships of OS₂ in this system. The relatively low activity of OS₂ in this model is probably linked to the resistance of the chick preparation to the different β -neurotoxins from Australian elapids (106), although we observed that OS₂ is highly neurotoxic when directly injected in chick brain (unpublished data).

Several group IA and IIA venom sPLA₂s which are catalytically active (D49) or inactive (K49) act as potent myotoxins that induce muscle necrosis by disrupting the integrity of the plasma membrane (14). The myotoxicity of group I and II sPLA₂s may depend on different structural determinants. The primary mechanism of action of both

active and inactive group II myotoxic sPLA₂s is independent of sPLA₂ activity and is likely to be due to basic and aromatic residues in the C-terminal region that allow the toxin to bind directly to phospholipids or yet ill-defined protein targets on the surface of skeletal muscle cells (14, 35, 36, 95, 108). On the other hand, all potent myotoxic group I sPLA₂s were found to lack the pancreatic loop and to have a cluster of surface residues including R15, V88, A100, N108, and cationic and hydrophobic residues at positions 83 and 109 (109). Here, we found that OS₂, but not OS₁, exerts myotoxic effects with histological and biochemical features similar to those of group I and II sPLA₂ myotoxins. OS₂ has most of the residues associated with myotoxicity in group I sPLA₂s and lacks the pancreatic loop, whereas OS₁ lacks V88 and has the pancreatic loop (Figure 1). Like myotoxic group II sPLA₂s, OS₂, but not OS₁, also has basic and aromatic residues in its C-terminal region (Figure 1), including W117 (Figures 1 and 2), which probably plays an important role (110, 111). The role of these residues and sPLA₂ activity could not be analyzed in detail, since all the OS₂ mutants, except K31L–R34S, appeared to lack myotoxic activity. Since most of the sPLA₂ mutants that have lost myotoxicity have also lost enzymatic activity, one might conclude at first sight that sPLA₂ activity is critical. However, the relationship is still unclear, since the central chimera is not myotoxic, while it has a high sPLA₂ activity. Since most OS₂ mutants bind to the M-type receptor, while they are no longer myotoxic, it is unlikely that this receptor is involved in myotoxicity, at least in the mouse. It is also unlikely, because the receptor is expressed at very low levels in adult mouse skeletal muscle and because the very potent K49 myotoxins Ba-II and Ba-IV purified from *Bothrops asper* do not bind to the endogenous or cloned mouse M-type receptor (M.R., B.L., J.M.G., and G.L., unpublished data).

Overall, the same trend was observed for the different OS₂ mutants at the chick neuromuscular junction and for myotoxicity. The fact that all enzymatically inactive OS₂ mutants are inefficient suggests that catalytic activity is essential for the two types of toxicity. However, the fact that the central chimera is also inactive in both cases, while it has a high level of enzymatic activity, suggests that both catalytic activity and other cellular factor(s) are critical for peripheral neurotoxicity and myotoxicity, with the central region of OS₂ containing some key structural determinants. Importantly, these observations contrast with those observed for central neurotoxicity, where the H48Q mutant and the central and C-terminal chimeras were nearly as active as WT OS₂ (see above). This suggests that the neurotoxic mechanisms operating at the peripheral neuromuscular junction and those occurring in the brain may be distinct. Very recently, Montecucco et al. proposed that the enzymatic activity of different venom sPLA₂s is a key factor for peripheral neurotoxicity (40). This proposal was based on the fact that equimolar mixtures of lysophospholipids and fatty acids closely mimic the biological effects of venom sPLA₂s at the mouse neuromuscular junction. Unfortunately, no mutated neurotoxic sPLA₂s were used in this study. This proposal fits our results at the neuromuscular junction, but contrasts with our findings regarding central neurotoxicity. Since Montecucco et al. do not exclude the contribution of sPLA₂ receptors in their proposed mechanism of action (40), it may be possible that different receptors are targeted at the

peripheral and central nervous systems. The subtype of brain receptors identified with the C-terminal chimera would be one of these receptors.

Antimalarial and Anti-HIV Activity of OS₂ Mutants and Chimeras. Several venom sPLA₂s have been shown to kill the parasite *P. falciparum* during its intra-erythrocytic development (21) by an indirect mechanism mediated by hydrolysis of serum lipoproteins and production of lipid products which are toxic for the parasite (72). Our results with OS₂ and its mutants are in accordance with this mechanism. Indeed, the inhibitory potency of the mutants correlates with their relative decrease in enzymatic activity and is dependent on the presence of serum. In this case, OS₁ and the central chimera are as active as, or even more active than WT OS₂. Whether their effects are related to particular enzymatic properties (Table 3) toward serum lipoproteins requires further investigation.

Our previous studies have shown that several venom sPLA₂s can also inhibit HIV replication by a mechanism that is still ill-defined, but that may not be associated with catalytic activity, since catalytically inactive K-49 sPLA₂s can inhibit HIV, while sPLA₂ catalytic products and inhibitors do not block infection (20, 37). Among all the sPLA₂s tested, bee venom sPLA₂ appears to be the most potent with an IC₅₀ of 0.6 nM. WT OS₂ is about 60-fold less potent with an IC₅₀ of 35 nM, yet it is more potent than OS₁ (Table 2). This relatively low inhibitory activity has prevented a detailed analysis of the OS₂ antiviral structure–function relationships. Nevertheless, our results show that, as for central neurotoxicity, the N-terminal region, but not the central and C-terminal regions, of OS₂ is critical. At first sight, our results support the view that catalytic activity is important. However, the result with the C-terminal chimera, which is still potent against HIV, while it has a low enzymatic activity, supports the view that this mutant binds to a subtype of N-receptors which may be similar to the one identified in mouse brain.

Concluding Remarks. Our results demonstrate that some biological effects of OS₂ are due to its enzymatic activity (for example the antimalarial effect), while others, including central neurotoxicity, are more related to specific binding to target cells than enzymatic activity. The most obvious dissociation between enzymatic activity and central neurotoxicity was observed with the H48Q mutant and the C-terminal chimera. On the basis of the chimera approach, both the N- and C-terminal regions are important for catalytic activity, but only the N-terminal is critical for central neurotoxicity and anti-HIV activity. On the basis of G30S and D49K mutants, the Ca²⁺-loop appears important for all activities, although it is highly conserved among sPLA₂s. On the basis of the central chimera, the β -wing structure did not appear to play a key role in the molecular properties or biological activities of OS₂. Amazingly, no clear role has been so far attributed to this particular structure or to the surface residues located in the two large α -helices on the back of the sPLA₂ (gray scaffold in Figure 2). This suggests that these two regions play only a structural role, serving as a rigid scaffold on which the functionally important and flexible domains containing the key residues involved in the specific sPLA₂ functions are anchored (92). Finally, our findings may help to develop novel strategies to neutralize the toxicity of venom sPLA₂s (28) or to engineer sPLA₂s with more specific functions. It will be interesting to analyze

in particular (i) the effect of sPLA₂ competitive inhibitors or soluble M-type receptors on the biological effects of OS₂ and other sPLA₂s and (ii) the effects of peptides from the N-terminal α -helix or the Ca²⁺-loop for their neurotoxic or anti-HIV properties, or for their inhibition of the sPLA₂ action, as these peptides may be agonists or antagonists. This strategy has already been used for myotoxic K49 sPLA₂s where short peptides from the C-terminal region appeared to be myotoxic (36, 112).

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