ELECTROSTATICALLY NEUTRAL INTERFACIAL BINDING SURFACE TARGETS ZWITTERIONIC MEMBRANES

Ying H. Pan‡, Bao-Zhu Yu‡, Alan G. Singer§, Farideh Ghomashchi§, Gérard Lambeau¶, Michael H. Gelb§, Mahendra K. Jain§, and Brian J. Bahnson‡

From the ‡Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716, the §Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington 98195, and the ¶Institut de Pharmacologie Moléculaire et Cellulaire, CNRS-UPR 411, 660 route des Laculaes, Sophia Antipolis 06560 Valbonne, France

The crystal structure of human group X (hGX) secreted phospholipase A₂ (sPLA₂) has been solved to a resolution of 1.97 Å. As expected the protein fold is similar to previously reported sPLA₂ structures. The active site architecture, including the positions of the catalytic residues and the first and second shell water around the Ca²⁺ cofactor, are highly conserved and remarkably similar to the group IB and group IIA enzymes. Differences are seen in the structures following the (1–12)-N-terminal helix and at the C terminus. These regions are proposed to interact with the substrate membrane surface. The opening to the active site slot is considerably larger in hGX than in human group IIA sPLA₂. Furthermore, the electrostatic surface potential of the hGX interfacial-binding surface does not resemble that of the human group IIA sPLA₂; the former is highly neutral, whereas the latter is highly cationic. The cationic residues on this face of group IB and IIA enzymes have been implicated in membrane binding and in kcat allostery. In contrast, hGX does not show activation by the anionic charge at the lipid interface when acting on phospholipid vesicles or short-chain phospholipid micelles. Together, the crystal structure and kinetic results of hGX supports the conclusion that it is as active on zwitterionic as on anionic interfaces, and thus it is predicted to target the zwitterionic membrane surfaces of mammalian cells.

Recent molecular cloning studies have shown that mammals contain a large number of secreted phospholipases A₂ (sPLA₂).¹ 9 gene products in humans and 10 in mice (1, 2). These 14–18-kDa disulfide-rich proteins require Ca²⁺ as a catalytic cofactor for the hydrolysis of the sn-2 ester of glycerophospholipids at the interface. For the past 4 decades the 14-kDa pancreatic group IB sPLA₂ has been a paradigm for interfacial enzymes that exhibit processive turnover at the substrate interface (3–5). The other sPLA₂ family members are of interest because they often act in concert with intracellular 87-kDa phospholipase A₂ to liberate arachidonic acid from cellular phospholipids as the substrate for the eicosanoid pathways (6–8). We are now faced with the challenge of understanding the physiological functions of the full set of mammalian sPLA₂s. The fact that each of these enzymes has distinct expression profiles and tissue distributions argues that they do not have redundant functions, although such functions remain to be established. Also, the tissue distribution of a particular human sPLA₂ is often distinct from that of its mouse ortholog (1). Thus, one cannot always infer the function of a human sPLA₂ from genetic studies with sPLA₂-deficient mice. It appears that, coupled with biochemical characterization of their function, highly selective inhibitors for each sPLA₂ gene product could facilitate the search for their specific physiological functions.

A portion of the gene that codes for the human group X sPLA₂ (hGX) was identified in GenBank™, and the full-length sequence was obtained from a fetal lung cDNA library (9). This sPLA₂ contains a disulfide pattern that is a combination of some of the unique disulfides found in group I and II sPLA₂s. Furthermore, hGX lacks the loops that are characteristic of the pancreatic and elapid snake enzymes, and was therefore given the unique name group X (9). The mRNA for hGX was found in spleen, thymus, peripheral blood leukocytes, and to a lesser extent in pancreas, lung, and colon cells (9). Unlike the group IB and IIA sPLA₂s that display significant preference for vesicles of anionic phospholipids (10), hGX is able to hydrolyze phosphatidylcholine-rich vesicles at a rate comparable with its action on anionic phospholipid vesicles (11). As a result, hGX is able to potently release arachidonic acid when added exogenously to the phosphatidylcholine-rich extracellular face of the plasma membrane of a variety of mammalian cells (11–13). Based on this specificity profile, hGX has been implicated to play a critical role in the arachidonic acid-mediated cyclooxygenase-2 pathway during colon carcinogenesis (14). There are dramatically different affinities of the various sPLA₂s for membrane surfaces containing different phospholipids. This is likely to be controlled by the amino acid differences that lie on their interfacing binding surfaces (i-face) rather than the substrate affinity controlled by the active site residues. The i-face is the nearly planar surface of sPLA₂ that surrounds the opening to the active site slot. The later is a ~15 Å deep pocket.
of sPLA₂ where a single phospholipid molecule must enter to reach the catalytic residues necessary for ester bond hydrolysis (5, 15–17).

The goal of the present study was to obtain structural information of the hGX enzyme to provide clues into its substrate and interface specificity, mode of interfacial activation, and most importantly its physiological function. Here we report the 1.97-Å crystal structure of a ligand-free form of hGX as a prelude to understanding the i-face residues that allow its relatively high affinity for phosphatidylcholine-rich membranes. We also gain insight into structure-based design of inhibitors that are selective for this sPLA₂. Attempts were made to obtain the structure with and without the active-site inhibitor 1-hexadecyl-3-(trifluoroethyl)-sn-glycero-2-phosphomethanol (MJ33) (18). Two crystal forms were obtained that led to independent structures of hGX, both without bound inhibitor.

EXPERIMENTAL PROCEDURES

Expression and Purification of hGX—The hGX was purified by a new procedure that gives improved yield compared with our original procedure (11). Sulphonated fusion protein was dissolved to 10 mg/ml in 6 M guanidine-HCl, 50 mM Tris-HCl, pH 8.0, by stirring overnight at 4 °C. The sample was centrifuged at 4 °C at 12,000 rpm (SS-34 rotor) for 20 min to remove any undissolved protein. The resulting protein solution (4 ml) was added to the refolding buffer (50 mM Tris-HCl, pH 8.0, 0.9 M guanidine-HCl, 0.8 M NaCl, 10 mM CaCl₂, 5 mM freshly added cysteine, 5 mM lauryl sulfobetaine), and refolding was allowed to proceed as had been performed for human group IIIF sPLA₂ (19). The protein solution was concentrated to 40–50 ml in a stirred cell with a YM-10 membrane (Amicon) and then dialyzed against 20 mM sodium phosphate, pH 6.6, 5 mM lauryl sulfobetaine, and 1 mM methionine at 4 °C. Trypsin was added (0.5 μg/ml), and digestion was allowed to proceed at room temperature for 12–15 h. The enzymatic activity was increased about 30-fold following digestion. Phenylmethylsulfonylfluoride was added to 0.5 mM, and the solution was stored for several hours at 4 °C or at –20 °C for longer periods.

The trypsin-digested hGX was purified by ion-exchange HPLC on a Tosohas DEAE-3SW column (0.5 × 15 cm) equilibrated with 20 mM sodium phosphate, pH 6.7, 30% CH₃CN, 1 mM methionine (buffer C). The resulting protein solution (10 ml) was injected onto the HPLC column pre-equilibrated with buffer C and was developed with the following gradient: 12% solvent D (solvent C = 1.0 M NaCl, 10 mM CaCl₂, 10 mM Tris-HCl, pH 8.0) over 2 min, 12.5% over 48 min, and 20.6% over 4 min at 20.6% F over 4 min at 3 ml/min. The hGX peak eluted at 40–44 min. The HPLC fraction was neutralized by the addition of 10 μl of 2 M Tris base per 1 ml of HPLC fraction. The pooled fractions were concentrated to 60–70% of the initial volume on a rotary evaporator, and lauryl sulfobetaine was added to 5 mM. The solution was concentrated to the desired final concentration (2–10 mg/ml) using a Centriprep-10 and finally dialyzed against 20 mM Tris-HCl, pH 8.0, 1 mM methionine. The protein was judged to be >98% pure on a 15% SDS-PAGE gel. The observed Mr (electrospray mass spectrometry) was 13,614.7, compared with a calculated Mr of 13,615.5.

Crystallization and X-ray Data Collection—Protein crystal growth experiments were designed to obtain crystals of the Ca²⁺-coordinated hGX enzyme with and without the bound competitive inhibitor MJ33. Protein crystal screening of hGX was carried out using the hanging drop method (20) at 25 °C and commercially available matrices (Hampton Research). Hanging drops were made of hGX-co-crystallized in the presence of the inhibitor MJ33 by mixing 2.0 μl of a stock solution containing 15 mg/ml protein, 10 mM CaCl₂, and 3 mM MJ33 with 2.0 μl of a reservoir solution of 10 mM CaCl₂, 12% PEG 3500, 10% 2-methyl-2,4-pentanediol (MPD), 2% ethylene glycol, and 0.2 M Hepes buffer, pH 7.4. Rectangular shaped crystals appeared in 3 days and were allowed to grow for 3 weeks before data collection. Protein crystals were obtained from hGX without the inhibitor MJ33 under identical conditions as those listed above except that the reservoir solution was buffered at pH 7.6. In this case, tetragonal-shaped crystals appeared in 3 days and were allowed to grow for 3 weeks before data collection.

X-ray diffraction data sets were collected for two hGX crystal forms on a Rigaku RU-H3R rotating anode generator with a RAXIS IV image plate area detector. For the hGX crystal form with MJ33 present, two crystals were needed to collect a complete data set. Otherwise, both crystal forms were pretreated in a similar fashion. Each required the removal of 2.0 M lauryl sulfobetaine and then dialysis against 20 mM Tris-HCl, pH 8.0, 1 mM MJ33, resulting in the absence of MJ33. A group IIA sPLA₂ structure from Chinese cobra (Protein Data Bank number 1POB) was used as the initial search model (23). The cobra venom protein sequence was 29% identical and 39% similar to the hGX enzyme. Three independent solutions were found in the rotational search with a peak intensity almost twice above the noise level. The translation search gave a unique solution within the asymmetric unit and with a final correlation factor of 28.1% and R-factor of 46.9%. The refined hGX model was then used to solve the structure of the second crystal form (C222₁) grown with MJ33 present in the crystallization condition. This crystal form had two subunits of hGX in the asymmetric unit, and the translation search gave a final correlation factor of 59% and an R-factor of 38%.

Structural Data Processing—A standard refinement procedure using the program CNS (24) were successful in obtaining a reliable model in each crystal form. Iterative model improvement was done using the programs CHAIN (25) and O (26). In refining the structure in space group P2₁, the initial model obtained from molecular replacement was subjected to a round of rigid body, positional, individual temperature factor (B-factor), and simulated annealing (5000 K) refinement to 2.5 Å, resulting in an Rfree of 0.474 and an Rwork of 0.388. The correct sequence of hGX (gi:4505845) was introduced into the refinement model. After one round of positional and simulated annealing refinement the Rfree and Rwork was 0.415 and 0.335, respectively. With 483 solvent molecules built into the model and extending the resolution to 1.97 Å, the final Rfree was 0.232 and Rwork was 0.190 with good geometry. Refinement of the C222₁ crystal form was more straightforward, starting from the nearly final model from the P2₁ crystal form. The model for this crystal form was refined to a resolution of 2.09 Å, and included 248 waters added to the model, yielding an Rfree and Rwork of 0.252 and 0.211, respectively, with good geometry.

Catalytic Function and Interfacial Binding of hGX—These procedures were performed at 24 °C and pH 8.0 under conditions specified in the figure legends. Procedures already established for other secreted PLA₂ enzymes were used for the characterization of hGX, and the results unique to hGX were developed in detail (4, 5). For example, the initial rate of hydrolysis of DC₆PC was compared by the pH stat (27) and the fluorescent pH indicator dye methods (28). Formation of the premicellar aggregates with 1,2-dihexyglycero-sn-3-phosphocholine (DC₆PC-ether) was monitored as a change in the 90° scattering at 380 nm with an SLM-aminco fluorimeter.

RESULTS

Structural features of the 1.97-Å crystal structure of a ligand-free form of hGX are described and interpreted in relation to its functional characteristics. The statistics of the x-ray data collection and refinement of two crystal forms of hGX are summarized in Table I. As expected the protein fold of hGX was quite similar to previously reported sPLA₂ structures. The predicted disulfide-bonding pattern (9) that led to the classification of hGX as a group X enzyme has been confirmed to contain 8 disulfide bonds. Beyond predictions of a novel protein fold, other potential structural differences can be predicted by gazing at a sequence alignment of sPLA₂ family members, as shown in Fig. 1. The hGX enzyme was between 29 and 41% identical to other members of the 14-kDa family of sPLA₂ enzymes. Residues that were part of the active site were highly conserved, and as expected, the active site of the hGX structure was remarkably similar to the previously solved group IB (29) and group IIA (30, 31) enzymes. However, there were noticeable differences in the hGX structure, relative to the group IB and IIA enzymes, and in the i-face binding surface. The asso-
Table I

| X-ray data collection and refinement of hGX sPLA2 ligand free structures |
|---------------------------------------------------------------|
| hGX crystal form | hGX (no MJ33) | hGX (with MJ33) |
| Crystal data | | |
| Space group | P2₁ | C222₁ |
| Cell parameters | | |
| a (Å) | 51.2 | 139.1 |
| b (Å) | 62.1 | 145.1 |
| c (Å) | 70.4 | 28.1 |
| β (°) | 99.3 | | |
| Subunits/asymmetric unit | 3 | 2 |
| X-ray data | | |
| Total reflections | 337,721 | 184,444 |
| Unique reflections | 34,475 | 19,931 |
| Resolution limit (Å) | 1.90 | 2.00 |
| Completeness (%) | 99.8 | 95.8 |
| Rmerge (%) | 6.3 | 8.1 |
| Refinement | | |
| Resolution range (Å) | 6.0–1.97 | 6.0–2.09 |
| Rwork | 0.190 | 0.252 |
| Rfree | 0.232 | 0.211 |
| RMSD observed | | |
| Bond length (Å) | 0.005 | 0.005 |
| Angle (°) | 1.16 | 1.14 |
| Total protein atoms | 2835 | 1890 |
| Total water molecules | 481 | 259 |

* Rmerge = ΣIᵢ – Iᵢ/Iᵢ, where Iᵢ is the observed intensity and Iᵢ is the average intensity, the sums being taken over all symmetry related reflections.

** R-factor = Σ|Fᵢ – Fᵢ|/ΣFᵢ, where Fᵢ is the observed amplitude and Fᵢ is the calculated amplitude. Rfree is the equivalent of Rwork, except it is calculated for a randomly chosen set of reflections that were omitted (5%) from the refinement process (52).

Fig. 1. Sequence alignment of selected sPLA₂ proteins compared with the hGX enzyme. The sPLA₂ protein sequences compared with their sequence accession codes and Protein Data Bank codes for enzymes of known structure were: hGX (gi:4505845), hGIIA (gi:4505848, Protein Data Bank number 1POE (30)), human group IB (hGIB, gi:4505846), pGIB (gi:129414, Protein Data Bank number 1FXF, (17)), human group V (gi:4505852), human group IID (gi:6912595), human group IIE (gi:7657461), and human group IIF (gi:12383057). The other two known human sPLA₂ sequences, group III and group XII, were not included because their sequences differ dramatically to the sequences presented here. The percent identity (and similarity) of each sequence relative to hGX is indicated at the end of each sequence. This alignment was performed using PILEUP from the GCG software and displayed with the program BOXSHADE.

Crystallized active site slot provides significant insights into the functional differences between these paralogs. These structural observations provide a framework to understand the substrate preference and kinetic differences observed for hGX compared with the group IB and IIA enzymes.

Active Site Coordination of hGX—The active site of the hGX structure for crystals of both crystal forms (P2₁ and C222₁) are shown in Fig. 2. The ligand-free structure of hGX (Fig. 2A) shows an arrangement of side chains, calcium coordination, and bound waters that was virtually identical to the structures of group IB and IIA sPLA₂ structures without bound ligands (29, 30). Just as has been observed in the group IB and IIA structures, the catalytic Ca²⁺ has octahedral geometry with 7 coordinating oxygen ligands with Ca²⁺-oxygen distances that range from 2.3 to 2.6 Å. The Ca²⁺-binding loop was comprised

![Figure 2](image-url)
of three carbonyl oxygens from Phe-26, Gly-28, and Gly-30. The two carboxylate oxygens of Asp-47 share an equatorial coordination site to Ca$^{2+}$. The remaining positions were occupied by the axial water w12 and the equatorial water w5, often referred to as the catalytic water. The second sphere waters, w6 and w7, bridge the acid/base catalyst His-46 and the Ca$^{2+}$ ligands w5 and Asp-47. The conservation of an hGX active site including active site residues and mechanistically critical waters suggests a conserved mechanism of this class of sPLA$_2$ enzyme.

hGX Co-crystallized with MJ33 Yielded a Ligand-free Structure—Two crystal forms were obtained of hGX controlled by whether or not the inhibitor MJ33 was added. The crystal form grown in the absence of MJ33 was in the P2$_1$ space group and had 3 subunits of hGX in the asymmetric unit. The crystal form grown with MJ33 at a saturating concentration (3 mM) was in the space group C222$_1$, with 2 subunits in the asymmetric unit. However, this crystal form also yielded a ligand-free crystal structure. The active site of the hGX structure from crystals that were grown in the presence of MJ33 is shown in Fig. 2B. The electron density map (2$\Phi_a$ – $\Phi_c$) unequivocally identifies this as a ligand-free structure. Careful examination of $\Phi_a$ – $\Phi_c$, difference electron density maps confirmed the absence of a substrate-mimic ligand bound to the Ca$^{2+}$. Difference density was located farther from the bound Ca$^{2+}$, which led to the modeling of two molecules of MPD into the hGX active site (Fig. 2B). The position of these MPD molecules was very similar to the placement of MPD solvent molecules into the 0.97-Å crystal structure of bovine group IB sPLA$_2$, which was likewise ligand free (29).

Comparison of hGX to Group IB and IIA sPLA$_2$ Structures—As one would expect from the alignment of sPLA$_2$ sequences (Fig. 1), the main chain C$_\alpha$-trace of hGX resembles the group IIA structures more than the group IB pancreatic enzymes. An overlay of group X, IIA, and IB enzymes was shown in Fig. 3 highlighting some minor, yet potentially significant differences. The group IB enzymes have an insertion of 5 residues (amino acids 62–66, Fig. 1) relative to the hGX and human group IIA (hGIIA) sequences. This insertion is shown in Fig. 3A demonstrating the result of this difference by a significantly altered surface loop (amino acids 59–75) for the porcine group IB enzyme (pGIB) shown at the top of the figure. Tyr-69 of group IB enzymes binds with the sn-3 phosphate moiety of the substrate mimics (32–34). The analogous position in the group X and IIA enzymes contains a lysine residue at this position, which likewise interacts with the sn-3 phosphate moiety of an inhibitor complex with the hGIIA enzyme (30). It was also significant that the differences of this i-face surface loop greatly affect the size and shape of the sPLA$_2$ active site. The other notable difference was the C-terminal extension of group X and IIA structures relative to the group IB structures.

More subtle differences exist between hGX and the group IIA sPLA$_2$ structures. The i-face surface loop from amino acids 62 to 66, Fig. 1) relative to the hGX and human group IIA (hGIIA) sequences. The overlay shown in Fig. 3A demonstrates the result of this difference by a significantly altered surface loop (amino acids 59–75) for the porcine group IB enzyme (pGIB) shown at the top of the figure. Tyr-69 of group IB enzymes binds with the sn-3 phosphate moiety of the substrate mimics (32–34). The analogous position in the group X and IIA enzymes contains a lysine residue at this position, which likewise interacts with the sn-3 phosphate moiety of an inhibitor complex with the hGIIA enzyme (30). It was also significant that the differences of this i-face surface loop greatly affect the size and shape of the sPLA$_2$ active site. The other notable difference was the C-terminal extension of group X and IIA structures relative to the group IB structures.

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The critical functional features of sPLA2 are intricately con-
nyl chain
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view shown for each structure presents the i-face binding surface of the enzyme. The active sites shown in the center of each structure have
white
positive charge and
red
displayed color-coded onto a meshed van der Waals surface using the program GRASP (51), where red and blue represent a net negative and
as a useful model to predict potential differences in substrate
and inhibitor specificity (30). Although Cn overlays demon-
strate a very similar core structure, considerable differences exist on the i-face surface of these two enzymes, as well as the
entrance of their active site pockets.

Electrostatic Potential and van der Waals Surface of hGX—
The critical functional features of sPLA2 are intricately con-
trolled by how the enzyme interacts with its substrate inter-
face. Experimental approaches have led to a prediction of not
only which surface of the sPLA2 enzymes interact with the
interface, but additionally the specific nature of these interac-
tions (5, 16, 17, 35). An electrostatic potential and van der
Waals surface comparison of the i-face of hGX versus hGIIA is
shown in Fig. 4, which shows the most dramatic, and likely,
the most significant differences between these two enzymes.
The human synovial fluid group IIA enzyme displays an exten-
sively positive charged i-face surface. In contrast the hGX i-face
surface was mainly nonpolar in nature and therefore electro-
statically neutral. Another striking difference can be observed
(Fig. 4) from a comparison of the van der Waals surface of the
active site opening of ligand-free structures of hGX versus
hGIIA. The hGX active site was considerably more open, pos-
sibly indicating a functional need to accommodate substrates
with polyunsaturated sn-2 chains, such as arachidonate.
Among mammalian sPLA2s, hGX and mouse group X display
with polyunsaturated
arachido-

FIG. 4. Electrostatic surface potentials of hGX (A) and hGIIA (B) in a ligand-free form (Protein Data Bank number 1POD (30)). The
view shown for each structure presents the i-face binding surface of the enzyme. The active sites shown in the center of each structure have
noticeably different sizes and shapes. The sPLA2 model of each structure is displayed in stick format with CPK colors. The potential surface is
displayed color-coded onto a meshed van der Waals surface using the program GRASP (51), where red and blue represent a net negative and
positive charge and white represents overall neutral positions, respectively.

| Substrate            | $v_{\text{app}}$ (s$^{-1}$) | $K_m^{\text{app}}$ (mM) |
|----------------------|----------------------------|-------------------------|
| DMPM vesicles$^a$    | 10                         |                          |
| Dithio-DMPM vesicles$^a$ | 9                         |                          |
| DMPC vesicles$^a$    | 20                         |                          |
| DC$_3$PC monomer     | 20                         | 0.25                    |
| DC$_3$PC micelle in 1 molar NaCl | 45                  | 0.43                    |
| DC$_3$PC micelle in 4 molar NaCl | 34                 | 0.12                    |

$^a$ The apparent rate parameters were obtained by fitting the concentra-
tion dependence of the observed initial rate under the respective condi-
tions (41). Thus $V_{	ext{app}}$ is the initial rate at $X_i = 1$, and $K_m^{\text{app}}$ is the
affinity of the enzyme for the micellar interface. The fit parameters
$V_{	ext{app}}$ and $K_m^{\text{app}}$ have estimated errors of 30% or their reported values.

|$v_o$ is the observed initial rate for substrates at a mole fraction $X_i = 1$. Initial rates were reproducible to within an error range of 10% of each value.

High Activity of hGX on Zwitterionic Interfaces—In light of
the different i-face of hGX in comparison to that of hGIIA
(Fig. 4), it was of interest to explore the kinetic behavior of hGX
acting on zwitterionic interfaces. The apparent parameters for
the kinetic characterization of hGX on the various forms of
substrates are summarized in Table II. The hGX initial rate ($v_o$) of hydrolysis at $X_i = 1$ on dimyristoyl-sn-3-phospho-

$^a$ M. H. Gelb, unpublished data.
FIG. 5. The concentration dependence of the initial rate of hydrolysis of DC$_6$PC (CMC of 1.5 mM). The rate of hydrolysis was measured as a change in the fluorescence from a pH-indicator dye (28) for the rate in an unstirred solution (solid triangles), the rate in a stirred solution (filled squares), and rates measured by the pH stat (27) (open circles) method in a vigorously stirred solution.

and air bubbles (27). The maximum rate of hydrolysis below the critical micelle concentration (CMC) of DC$_6$PC was about 25 s$^{-1}$ compared with the rate of 45 s$^{-1}$ at the saturating concentration of DC$_6$PC (Fig. 5). Note that $V_{\text{max}}$ remains virtually the same in the presence of 4 mM NaCl even though $K_m$ decreases by a factor of three. The decrease in $K_m$ was most likely because of the salt-induced hydrophobic effect, which increases the affinity of the enzyme for the interface and also lowers the CMC of the micellar substrate (41). On the other hand, when comparing rates with 1 mM pH indicator dye (28) vs. 4 mM NaCl added, there was only a marginal change in the value of $V_{\text{max}}$. At high concentrations of NaCl the phosphatidylcholine interface becomes negatively charged because of a preferential partitioning of chloride anions (42). This increase in negative charge does not appear to affect the hGX chemical step. This was consistent with kinetic data with phospholipid vesicles (Table II) showing that the rate of hydrolysis by hGX was not influenced significantly by the anionic charge at the interface.

Several features of these results are noteworthy especially in relation to the behavior seen with pGIB (41). The rate of hydrolysis below the CMC of DC$_6$PC was significant: 20 s$^{-1}$ with hGX compared with <0.03 s$^{-1}$ with pGIB. Similarly, as summarized in Table II the rate enhancement above the CMC is only 2-fold for hGX, and the rate of hydrolysis of the micellar DC$_6$PC does not change in the presence of 4 mM NaCl. In contrast, with pGIB the rate above the CMC was 15 s$^{-1}$, and a further 50-fold rate enhancement was seen in the presence of 4 mM NaCl.

The origin of a significant rate of hydrolysis of monodisperse DC$_6$PC by hGX was examined further. A small difference between the rates in the stirred and unstirred solution (Fig. 5) suggests that the contribution of the rates from the reaction on the walls of the reaction vessel was small compared with the rate of hydrolysis of the monodisperse substrate. This was consistent with the possibility of the formation of a monodisperse hGX-DC$_6$PC complex in aqueous solution. However, it was also possible that hGX forms premicellar aggregates with monodisperse substrate in which the turnover occurs via an interfaceal surfactant of the Michaelis complex $E^*S$. In fact, results summarized in Fig. 6 show that premicellar aggregates were formed between hGX and the nonhydrolyzable DC$_6$PC-ether amphiphile. The DC$_6$PC-ether in the absence of enzyme remains monodisperse up to 4 mM. The increase in the intensity of the scattered light in the presence of hGX and monodisperse DC$_6$PC-ether indicates the formation of premicellar aggregates. Moreover, the scattering increase was larger in the presence of calcium than without calcium added. The initial $E$ to $E^*$ step does not require Ca$^{2+}$. However, sPLA$_2$ enzymes require a bound Ca$^{2+}$ to bind substrates or substrate mimics. Therefore, according to the detailed balance condition (5, 41), the apparent tendency to form a premicellar aggregate would increase in the presence of Ca$^{2+}$. The observed formation of such premicellar aggregates makes it quite likely that the hGX catalyzed hydrolysis of substrates proceeds at the interface via an interfacial active $E^*$ form of the enzyme even though the bulk of the substrate amphiphile remains monodisperse in the reaction mixture. Under this scenario, the reaction progress can proceed at a rapid rate at such an interface, so long as the rate of substrate replenishment is rapid (5, 41).

**TABLE III**

| Inhibitor$^a$ | $X_{50}$ $^b$ |
|--------------|-------------|
| MJ33         | 0.052       |
| MJ50         | 0.03        |
| Oleoylamide (cis) | 0.011   |
| Elaidoylamide (trans) | 0.0055  |
| Masticadienoic acid | 0.055  |
| HK40         | 0.022       |
| PG-urethcr   | 0.0058      |
| PC-urethcr   | 0.0017      |
| Zn$^{2+}$    | 2.5 $\mu$M |
| MPD          | 300 mm      |

$^a$ MJ50, 1-hexadecyl-3-(trichloroethyl)-sn-glycero-2-phosphethanol; HK40, 1-deoxy-1-octylthio-2-heptylphosphonyl-sn-glycero-3-phosphoethanol; masticadienoic acid (53); PC-urethcr, (R)-2-O-decanoylamino-octanol-1-O-phosphocholine; PG-urethcr, (R)-2-O-decanoylamino-octanol-1-O-phosphoglycol; PC-urethcr and PG-urethcr were kindly provided by Professor de Haas (Utrecht).

$^b$ The inhibitor concentrations that produce half-activity of DMPM hydrolysis, $IC_{50}$, are reported in micromolar for Zn$^{2+}$ and MPD. For the other inhibitors, which are assumed to be completely partitioned in the DMPM interface, $X_{50}$ is the mole fraction of the inhibitor that gives half-activity (18). The reported values of $X_{50}$ are reproducible to within 10% of each value.

**DISCUSSION**

As is apparent from other sPLA$_2$ structures, the hGX structure reaffirms a clear dissection of the active site and the i-face.
Operationally, the difference is useful for assigning independent roles for the structural features involved in the events of the catalytic cycle and the binding of the enzyme to the interface. Results at hand show that while retaining the architecture of the catalytic site, the i-face of hGX was significantly different from several other sPLA₂. The hGX PLA₂ has the ability to form premicellar aggregates with zwitterionic amphiphiles, and it hydrolyzes zwitterionic and anionic phospholipid vesicles with similar catalytic efficiency (Table II). In these respects, hGX behaves like cobra venom sPLA₂ (39, 43) and is the first mammalian sPLA₂ shown to have these properties. The rate of hydrolysis of DC₂PC by hGX (Fig. 5) and cobra venom sPLA₂ (39, 43) shows only a modest increase when the substrate concentration is increased above its CMC. This initially gives an impression that these enzymes do not exhibit the classical interfacial activation that is the hallmark of other sPLA₂ such as pGIB (3). However, the lack of a large increase in rate above the CMC value of DC₂PC is presumably because, well below the CMC value of the substrate, hGX forms its own interface as a protein-lipid premicellar aggregate (Fig. 6). The structure of the hGX enzyme has a more pronounced hydrophobic patch than the group IB and IIA enzyme. This surface is likely critical for premicellar interaction with the hydrophobic tails of the DC₂PC substrate. Additionally, the dramatically different electrostatic potentials of the hGIIA and hGX i-faces (Fig. 4) provide a working hypothesis for the structural basis for the observed interfacial binding selectivity with preformed interface (Table II) as well as for the formation of premicellar aggregates. These structural differences observed between the hGX and hGIIA i-face residues likewise set the stage for an investigation of their functional differences by detailed site-directed mutagenesis studies.

The effects of the interface and the anionic charge on the catalytic efficiency of hGX versus pGIB and hGIIA were dramatically different. Under optimal conditions with optimal substrate interface to bind all the enzyme, the turnover rate at the maximum substrate mole fraction Xₐ* = 1 is about 20-fold higher for pGIB (36, 41), and is 4-fold higher for hGIIA (37) than that for hGX. At this stage we cannot ascertain if the lower rate with hGX was because of a higher Kₘ for an impaired kₐcat. However, it was of interest to note that the affinity of hGX for the various inhibitors (Table III) was of comparable magnitude to that for other sPLA₂. If Kₘ for the substrate was controlled through the same interactions as the binding of inhibitors, the change in the rate at Xₐ = 1 would suggest an impaired kₐcat for hGX. A compromised chemical step is also indicated by the observation that the hGX enzyme does not kinetically distinguish between the oxy- or thio-dimyrysto-sn-3-phosphomethanol (Table II). This contrasts with the reported dimyrysto-sn-3-phosphomethanol oxy/thio ratio of about 12 for pGIB, which is thought to have a rate-liming chemical step (44).

The lack of the inhibitor MJ33 in the hGX C222 crystal form was initially disconcerting. However, a simple explanation is that the active site of hGX has been out competed by the MPD solvent molecules instead of the MJ33 inhibitor. Further attempts are in progress to obtain structures of the hGX enzyme with bound active site ligands. In the absence of such structures, structure alignments with other inhibitor-bound sPLA₂ structures can be used to predict potential differences. Specifically, this comparison has been done between the hGX from this work and the hGIIA bound to a tetraedral mimic inhibitor (30 enzymes as shown in Fig. 3R. Although the structure of the active site of sPLA₂ is highly conserved, the residues that line the active site slot are not identical among these enzymes. Thus, it has been possible to discover active site inhibitors that display significant selectivity among various sPLA₂. For example, MJ33 is a potent inhibitor of pGIB (18) but is not an inhibitor of human group IIA sPLA₂ (37). Likewise, the indole-3-acetamide analog LY311727 (45) and related analogs (46, 47) bind about 2 orders of magnitude tighter to hGIIA and human group IIE sPLA₂ than to hGX and human group V sPLA₂. Because the x-ray structures of three of these indole-3-acetamide analogs bound to hGIIA have been determined (48), the variation in the active site residues that contact the inhibitor among the different mammalian sPLA₂ can be compared. Most of these residues are invariant among the different enzymes, but two notable exceptions are seen. Leu-5 and Leu-98 of hGX are conserved in human group V sPLA₂, but are replaced by phenylalanines in the hGIIA and human group IIE sPLA₂. In the hGIIA structure, Phe-5 and Phe-98 are well packed against the face of the indole ring of the bound inhibitor. Substitution by leucine in hGX and human group V sPLA₂ would be expected to leave a gap between the enzyme and inhibitor that would not be ideal for high affinity binding. The x-ray structure of hGX reported here is expected to facilitate the development of potent and selective inhibitors of hGX.

To understand the determinants of potent inhibitors and substrate specificity there are additional issues to consider beyond a comparison of active site residues and the charged nature of the i-face of the enzymes. In general, sPLA₂ enzymes have been suggested to undergo structural changes upon binding an interface (4). It is speculated that many of these changes are mediated by surface loops that are part of the i-face of the enzyme. The observation of both cis- and trans-proline at position 14 of hGX may be of significance to determining interfacial activity and/or specificity. The cis/trans observation suggests that the proline peptide bond is at equilibrium between the cis- and trans-conformation. The thermodynamics and rates of cis-/trans-proline isomerization have been thoroughly studied for nonenzymatic and enzymatic reactions (49). Even on the interface, the rates of isomerization are likely significantly slower than the time scale of the reaction. However, for enzymes like the hGX PLA₂, it may be that one form may be uniquely stabilized in a productive interface bound mode.

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