Gossypol Modification of Ala-1 of Secreted Phospholipase A_2: A Probe for the Kinetic Effects of Sulfate Glycoconjugates[†]

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Received December 4, 1996; Revised Manuscript Received June 19, 1997[⊗]

ABSTRACT: Gossypol is shown to covalently modify secreted phospholipase A_2 (PLA2) in the aqueous phase, but not at the interface. A rapid initial noncovalent binding of gossypol is followed by a slow covalent modification of the α-amino group of Ala-1 by stoichiometric amounts of gossypol. The rate of modification increases in the presence of calcium, but occupancy of the substrate binding site does not alter the rate. Pancreatic PLA2 is modified at the α-amino group of the N terminus to form a Schiff base, which can be stabilized by reduction with borohydride. Residual activity of the gossypol-modified PLA2 from several different sources is about 10%, indicative of impaired catalytic turnover. The half-time for the inactivation is about 10 min, and it is more than 100-fold longer for PLA2 at the interface. Gossypol promotes binding of PLA2 to the interface, and the binding of PLA2 to the interface promotes only the noncovalent binding of gossypol, but not the covalent modification. Gossypol, in conjunction with spectroscopic and kinetic protocols, is used to characterize the kinetic effects of sulfated glycoconjugates, heparin and artery wall proteoglycans, with human inflammatory and pancreatic PLA2.[1] The conjugates do not interfere with the binding of PLA2 to the interface or with the catalytic cycle at the interface. The conjugates do not influence the kinetics of modification of PLA2 by gossypol in the aqueous phase, and the enzyme at the interface is not modified in the presence of the conjugates. The conjugates bind to PLA2 at the interface with only a modest effect on the interfacial catalytic turnover without dislodging the bound enzyme. Complex kinetic effects induced by the conjugates are shown to be due to sequestration of PLA2 in the aqueous phase as a high-molecular mass complex, which dissociates with added NaCl.

The enzymes of 14 kDa phospholipase A_2 (PLA2) family are secreted from pancreas, venoms, and inflammatory cells in response to a variety of stimuli. For example, elevated levels of hiPLA2 in the circulation are associated with inflammatory conditions (Vadas, 1993), including pancreatitis (Nevalainnen & Gronroos, 1994), and certain surgical procedures involving heparin administration such as cardiopulmonary bypass (Nakamura et al., 1995). Metabolic roles and biochemical functions of interfacial enzymes in general, and of PLA2 in particular (Gelb et al., 1995), are difficult to evaluate in terms of the underlying kinetic mechanisms because observed activities of interfacial enzymes are modulated by factors not directly associated with the catalytic turnover cycle (Verheij et al., 1981; Jain & Berg, 1989). To understand possible mechanisms through which the activity of PLA2s is controlled and regulated, and to identify possible kinetic artifacts, we (Jain et al., 1995) have developed protocols and controls to characterize interfacial kinetics as

$$E \rightleftarrows E^* + S \rightleftarrows E^*S \rightarrow E^* + P \qquad (1)$$

A key feature of this minimal kinetic scheme for interfacial catalysis is the fact that the fraction of the enzyme bound to the interface (E to E* equilibrium) determines the effective rate of catalytic turnover (Berg et al., 1991), because changes in the E to E* equilibrium change the residence time of the enzyme at the interface (Jain & Berg, 1989). In addition, a slower rate of substrate replenishment on the enzyme-containing interface, relative to the rate-limiting chemical step, also leads to anomalous kinetic effects with mixed micelles (Jain et al., 1993a). If the exchange equilibria are constrained, it is possible to obtain kinetic results useful for unequivocal mechanistic interpretation (Jain et al., 1995). For example, if substrate and products do not exchange and if the binding of enzyme to the interface is of high affinity, the E to E* step becomes a pre-steady state step for the interfacial catalytic turnover in the highly processive scooting mode, where only the substrate on the enzyme-containing

[†] This work was supported by the NIH (GM29703 to M.K.J.).

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[⊗] Abstract published in *Advance ACS Abstracts,* September 15, 1997.

[1] Abbreviations: AMPA, peramidinated PLA2 from bovine pancreas; deoxy-LPC, 1-hexadecylpropanediol-3-phosphocholine; DC_nPC, 1,2-diacylglycero-*sn*-3-phosphocholine; DC_8PM, 1,2-dioctanoylglycero-*sn*-3-phosphomethanol; DMPM, 1,2-dimyristoylglycero-*sn*-3-phosphomethanol; DTPM, 1,2-ditetradecylglycero-*sn*-3-phosphomethanol; DNS-DTPE, *N*-dansylated 1,2-ditetradecylglycero-*rac*-3-phosphoethanolamine; g-PLA2, pig pancreatic PLA2 modified with gossypol and then reduced with borohydride; 2H-GPC, 2-hexadecylglycerol-*rac*-3-phosphocholine; hiPLA2, phospholipase A_2 secreted by human inflammatory cells; HPC, hexadecyl-1-phosphocholine; hsPG-I and -II, heparan sulfate-conjugated proteoglycans from human aorta; i-face, face of PLA2 that interacts with the interface during catalysis; iso-ppPLA2, natural isozyme of ppPLA2; MJ33, 1-hexadecyl-3-(trifluoroethyl)glycero-*rac*-2-phosphomethanol; MJ72, 1-octyl-3-(trifluoroethyl)glycero-*sn*-2-phosphomethanol; PLA2, secreted phospholipase A_2; ppPLA2, phospholipase A_2 from pig pancreas; PNBr, *p*-nitrophenacyl bromide (2-bromo-4′-nitroacetophenone).

S0006-2960(96)02972-8 CCC: $14.00

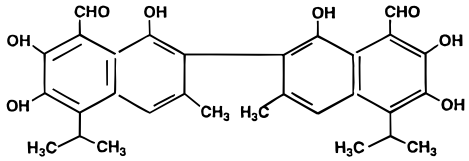


FIGURE 1: Gossypol.

Table 1: Half-Time of Inactivation and Residual Activity of PLA2s by Gossypol in a 1:8 Mole Ratio

source	half-time (min)	residual activity (%)
pig pancreas	6	22
pig AMPA	15	17
bovine PLA2	15	30
bovine AMPA	9.4	6
pig A1G AMPA	3	1
A. halys blomhoffi	12	7
bee venom	8.5	10
hiPLA2	1.2	10
V3W hiPLA2	2	10

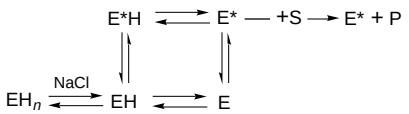
vesicles is hydrolyzed during the course of the reaction progress. This permits determination of the primary rate and equilibrium constants for the catalytic turnover (Berg et al., 1991; Jain et al., 1995), useful for interpretation of observed kinetics (Jain & Berg, 1989; Gelb et al., 1995). In this paper, we use such analysis to resolve complex kinetic effects of gossypol and sulfated glycoconjugates on PLA2.

(a) First (Figures 1−5 and Tables 1 and 2), we show that a stoichiometric amount of gossypol inactivates PLA2 in the aqueous phase by covalent modification of the α-amino group at the N terminus. The modification of PLA2 (E) by gossypol (G) is a two-step process where the initial rapid noncovalent binding to form E·G is followed by Schiff base (E═G) formation:

$$E + G \overset{\text{(fast)}}{\rightleftharpoons} E{\bullet}G \overset{\text{(slow)}}{\rightleftharpoons} E{=}G + NaBH_4 \xrightarrow{\text{(irreversible)}} EG \text{ (or g-PLA2)} \quad (2)$$

The Schiff base reduced with borohydride gives a stable modified enzyme with about 5% residual activity. Since PLA2 at the interface is not inactivated, gossypol is a useful chemical probe for monitoring binding of the enzyme to the interface.

(b) In the rest of this paper, we develop a kinetic basis for the apparent inhibition of PLA2-catalyzed hydrolysis under certain conditions by sulfated glycoconjugates. The conjugates do not significantly change the kinetics of inactivation by gossypol. Complex effects of heparin and conjugates on the PLA2 kinetics are rationalized in terms of the following equilibria:



(3)

Binding of heparin to PLA2 at the interface to form E*H has only a modest effect on the intrinsic interfacial catalytic turnover (last step). On the other hand, PLA2 preincubated with heparin does not show a significant turnover. The origin of this apparent inhibition is due to the aggregation tendency of the heparin−enzyme complex in aqueous phase, EH, to form a stable high-molecular mass complex, EH_n, which makes PLA2 virtually inaccessible for interfacial catalytic turnover.

Table 2: Half-Times for the Modification[a] of ppPLA2 by PNBr and Gossypol

experiment	conditions	PNBr *t* (min)	gossypol *t* (min)	RA (%)	form
1.	13 mM EGTA	3	55.4	<10	E
2.	5 mM Ca	100[b]	7	<10	E·Ca
3.	1.6 mM deoxy-LPC	7	>1000	80	E*
4.	Ca + 1.6 mM dexoy-LPC	100[b]	>1000	80	E*·Ca
5.	1.6 mM 2H-GPC	5	>1000	80	E*
6.	2H-GPC + Ca	100[b]	>1000	80	E*·Ca
7.	0.1 mM PC8-ether	100[b]	10	<20	E·Ca·L
8.	8 μM MJ33	14	5	80	E·Ca·L
9.	0.2 mM MJ72	15	10	80	E·Ca·L
10.	3 mM PC8-ether		185	50	E*·Ca·L
11.	0.1 mM PC8-ether + 1 μM MJ33	43	>1000	80	E*·Ca·L
12.	0.1 mM PC8-ether + 5 μM MJ72	30	>1000	70	E*·Ca·L
13.	deoxy-LPC+ 5 μM MJ33	80	>1000	80	E*·Ca·L

[a] Gossypol:PLA2 mole ratio of 11; PNBr:E mole ratio of 5000. RA (residual activity) is for the gossypol-modified enzyme, obtained from curve fitting (Figure 2). [b] The values are at 5 mM Ca. In experiments 8−13, the calcium concentration is 0.5 mM.

EXPERIMENTAL PROCEDURES

Recombinant hiPLA2 expressed in a Chinese hamster ovary cell line was kindly provided by J. Browning (Biogen, Cambridge). The V3W mutant of hiPLA2 was constructed and characterized as described (Othman et al., 1996). AMPA and semisynthetic Ala-1-Gly AMPA, the peramidinated derivatives of PLA2, were provided by A. Slotboom (Utrecht). ppPLA2 (Jain et al., 1991b), pro-ppPLA2 and iso-ppPLA2 (Jain et al., 1988), DMPM (Jain et al., 1986), MJ33 (Jain et al., 1991b), 2H-GPC (Jain et al., 1991a), DNS-DTPE (Jain & Vaz, 1987), DC_8PM, DC_8PM-ether, and DC_8PC-ether (Jain et al., 1986; Rogers et al., 1992, 1996) were prepared as described. DC_nPC was from Avanti.

Chondroitin sulfate A (Sigma C7571), 4% heparin−agarose (type 1), and three grades of heparin from pig mucosa were obtained from Sigma: 1-A (H3393), unbleached crude (H5515), and low-molecular mass (LMW, H3400). A sample of higher-molecular mass (HMW) heparin was kindly provided by J. Harmony. Several batches of hsPG-I and hsPG-II from human aorta, prepared as described by Hurt-Camejo et al. (1990), were kindly provided by F. Lopez (IVIC, Caracas). Typically, the hexuronate content of hsPG-I was about 30 wt %, and that of hsPG-II was 60 wt %. The concentrations are given on the protein mass basis. Qualitatively, the behavior of heparin and hsPGs was comparable in assays of the type shown in Figure 7 and Table 4; however, most of the studies were carried out with hsPG-I and LMW-heparin (H3400 from Sigma). Note that both of these preparations are microscopically heterogeneous. Heparins are sulfated oligosaccharides secreted by certain inflammatory cells. On the other hand, arterial wall hsPGs contain somewhat different sulfated oligosaccharide heparan repeat units conjugated to peptide chains. The peptidoglycans are solubilized during isolation; however, they tend to form insoluble aggregates on repeated lyophilization or freezing−thawing cycles.

Table 3: Characteristic Equilibrium Parameters[a] for the Complexes of V3W hiPLA2

parameter	V3W hiPLA2	ppPLA2	iso-PLA2
I_{50}(HMW-heparin) (μg)	3 (0.7)	9 (0.75)	810 (0.72)
I_{50}(NaCl) (mM)	350	20	25
I_{50}(3K-heparin) (μg)	3.2 (0.75)	13 (0.73)	800 (0.73)
I_{50}(NaCl) (mM)	330	25	30
K_{SV} (M^{-1})			
E alone	11.3	6.5	10.9
E + heparin	2.6	3.75	9.4
E + hsPG-I	3.2	4.4	7.8
E + DTPM (E*L)	0.7	0.3	0.4

[a] I_{50}(heparin) is the concentration of heparin (micrograms per 1.4 mL) for a 50% change in the fluorescence obtained as in Figure 10A. I_{50}(NaCl) is the concentration of NaCl (millimolar) for a 50% increase in the fluorescence as in Figure 10B. K_{SV} is the Stern−Volmer constant for quenching acrylamide.

Table 4: Elution Times[a] (Minutes) for Heparin on a TSK-250 HPLC Column

	no added salt		with 0.48 M NaCl	
heparin	alone	with ppPLA2[b]	alone	with ppPLA2[b]
unbleached	5.0	5.0	7.4/11.15	10.3
1-A	5.14	5.2	7.26	10.3
LMW	5.7	5.4	9.9	10.4
HMW	4.74	5.1	8.65	10.3

[a] Elution was carried out with a flow rate of 1 mL/min with 0.5 mM $CaCl_2$ in 5 mM phosphate buffer at pH 7.0. [b] ppPLA2 (complex) elution times.

Phospholipid dispersions were prepared in water at concentrations of 10−30 mg/mL, and the stock solution was diluted in an appropriate medium. Kinetic measurements by pH-stat titration (Radiometer or Metrohm) were carried out in 4 mL of 1 mM NaCl and 0.5 mM $CaCl_2$ at pH 8.0 in polypropylene cups (Jain et al., 1986). The kinetic basis and experimental protocols for interfacial catalysis in the highly processive scooting mode by PLA2 (Berg et al., 1991; Jain et al., 1995) have been described for the reaction progress on DMPM vesicles by the pH-stat method (Jain et al., 1986; Berg et al., 1991), kinetic characterization of competitive inhibition (Jain et al., 1991b, 1995), determination of the equilibrium dissociation constants of ligand bound to PLA2 at the interface by the protection from alkylation of the active site histidine by ligand (Jain et al., 1991a), and measurement of residual PLA2 activity in the covalent modification experiments with fluorescent substrate (Yu et al., 1993). Binding of PLA2 to the interface was monitored as a change in the intrinsic fluorescence emission from Trp-3 of PLA2 (Jain et al., 1982, 1986; Ramirez & Jain, 1991) or as the energy transfer to a dansyl fluorophore localized in the interface (Jain & Vaz, 1987). Fluorescence measurements were carried out on a SLM-Aminco AB2 apparatus with slit widths of 4 nm each. UV−visible absorbance measurements were carried out on an HP8452 diode array spectrophotometer. Unless indicated otherwise, all spectroscopic measurements were carried out at pH 8.0 in 10 mM Tris-HCl at 23−24 °C. Specific conditions used for these measurements are given in the figure legends. On the basis of standard deviations, uncertainty in primary experimental data is 10%, and 30% in the derived parameters.

Modification of PLA2 by Gossypol. The time course of covalent modification of PLA2 by gossypol was monitored in an incubation mixture containing varying mole ratios of gossypol to PLA2 (typically 0.2−0.4 μM enzyme) in 0.1 M cacodylate buffer with 0.1 M NaCl at pH 7.3 and 23 °C. For certain studies, the reaction mixture also contained calcium, deoxy-LPC, and competitive inhibitors of PLA2. The progress of inactivation by gossypol or PNBr was monitored by measuring the residual PLA2 activity with a fluorescent substrate (Yu et al., 1993). The half-time for inactivation was obtained with a nonlinear fit of the residual activity to a single- or double-exponential decay. Spectral changes in the initial phase of the inactivation of PLA2 by gossypol were similar to those observed with the reaction products of gossypol with polylysine or phenylalanine.

Characterization of g-PLA2, the Peramidinated ppPLA2 Covalently Modified by Gossypol. g-PLA2 was prepared by incubating 2 mg of bovine AMPA with a stoichiometric amount of gossypol (1:1.2 mole ratio) in 0.4 mL of 10 mM HEPES and 0.5 mM $CaCl_2$ at pH 8.0 and 23 °C. After 3 h, the mixture was cooled in an ice bath and treated with a 100-fold molar excess of sodium borohydride with stirring for 1 h. The mixture was dialyzed against the same buffer and then concentrated by ultrafiltration (Novacell with a 1K cutoff filter). It was purified by HPLC on a Vydec-C4 column in a 0 to 85% acetonitrile linear gradient (30 min at 1 mL/min) in 0.1% trifluoroacetic acid in water. The modified enzyme, g-PLA2, eluted at 35.9 min, compared to 29.9 min for the unmodified enzyme. When the reaction mixture without reduction with borohydride was applied to the HPLC column, the time at which AMPA eluted was unchanged due to reversal of the Schiff's base formed in the first step (eq 2). Control runs without gossypol showed that borohydride reduction and related manipulations had little effect on the elution profile or activity of AMPA.

The homogeneity of g-PLA2 obtained from AMPA was further checked by FPLC on an anionic MONO-S column where it eluted in 6 min (compared to 15 min for AMPA) in a 0 to 0.8 M NaCl gradient in 10 mM acetate buffer at pH 5.0. The molar extinction coefficient of g-PLA2 was 85 680 M^{-1} cm^{-1} at 260 nm; $E_{1\%} = 69$ at 260 nm and $E_{1\%} = 29.6$ at 280 nm, compared to the $E_{1\%}$ value of 13 at 280 nm for AMPA. The stability of g-PLA2 during dialysis and HPLC suggests that after reduction a stable linkage is formed between a gossypol molecule and a PLA2 molecule. The catalytic activity of g-PLA2 was 2−10% of the activity of AMPA in the various assays (Jain et al., 1991a, 1995; Yu et al., 1993); however, g-PLA2 was not kinetically characterized further.

Gel Filtration Studies. HPLC measurements were carried out on ISCO or Rainin hardware with appropriate columns. The TSK-250 gel permeation column gave very reproducible results. TSK-4000 and GPC-100 columns also gave comparable results under certain conditions, but could not be used under the whole range of conditions of interest. Although the matrix of the TSK columns is anionic and shows ion exchange properties, this was not a serious problem for monitoring the molecular sieving characteristics because heparins are anionic. Retention times for PLA2 and heparin on the TSK-250 column were monitored as absorbance (at 220 nm) and fluorescence (excitation at 280 nm with emission at 345 nm). All samples of heparins showed a weak UV absorbance with no detectable fluorescence, whereas PLA2, other than hiPLA2, gave the absorbance and fluorescence signals confirmed by the activity assay. Typically, the column was equilibrated at room temperature with 0.5 mM $CaCl_2$ and 5 mM KH_2PO_4 buffer at pH 7.0. Elution was carried out in the same buffer after injection of up to

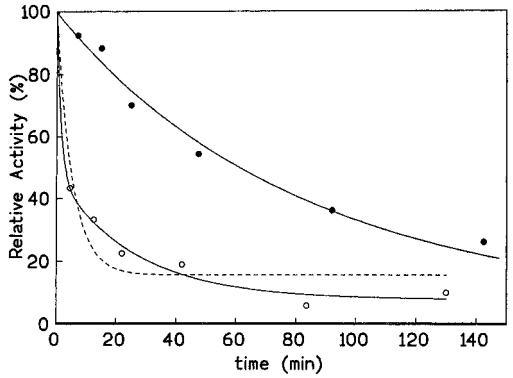


FIGURE 2: Time course for the inactivation of ppPLA2 by gossypol in the presence of 0.5 mM (open circles) or absence (closed circles) of calcium. The incubation mixture (30 μL) contained 100 mM cacodylate buffer at pH 7.3 and 23 °C with 0.2 μM ppPLA2 and 1.6 μM gossypol. The dashed line is a single-exponential fit, and the full line is a double-exponential fit for the data in the presence of calcium. Values of the inactivation half-times reported in this paper are those obtained with a single-exponential fit and are therefore approximate. Note that a single-exponential fit is adequate with AMPA (results not shown; however, see the text for a discussion).

20 μg of PLA2 preincubated with the indicated amount of heparin in 20 μL of buffer. The column was calibrated with several proteins at an elution rate of 1 mL/min with the uncertainty in the retention times of less than 0.1 min: 2000 kDa blue dextran (4.64 min), 440 kDa ferritin (6.2 min), 158 kDa aldolase (5.2 min), 66 kDa bovine serum albumin (7.88 min), 18.4 kDa lactoglobulin (9 min), 13.9 kDa ppPLA2 and other PLA2s (10.3 min), and 12.4 kDa cytochrome *c* (11.2 min). Comparable studies could not be carried out with hsPGs because they show a strong fluorescence, tend to aggregate, and have a broad distribution of molecular species in the 10−100 kDa range.

RESULTS

As outlined in the introductory section, this section describes two sets of studies. First, we show that inactivation of ppPLA2 by gossypol is due to covalent modification of the α-amino group of Ala-1 (eq 2). The fact that the modification is not seen with PLA2 bound to the interface is consistent with the consensus that the N terminus is a part of the face of PLA2 in contact with the substrate interface during the catalytic turnover (Jain & Maliwal, 1993), which we call the i-face (Ramirez & Jain, 1991). In the second part, we use gossypol as a probe, in conjunction with other protocols, to show that sulfated glycoconjugates bind to a site on PLA2 that is not the i-face or the catalytic site.

Kinetics of Inactivation of ppPLA2 by Gossypol. As shown in Figure 2, the loss of activity of ppPLA2 incubated with gossypol is time-dependent. About 70% of the inactivation of the native enzyme is adequately described by a single-exponential decay, and the balance can be fitted to a second exponential, suggesting that additional groups are modified at a somewhat slower rate in the presence of excess gossypol (see below). These results suggest that modification of one or more amino groups may be responsible for the reduced PLA2 activity. As shown in Figure 2, a rapid single-exponential decay completely describes the inactivation of bovine AMPA in which only the N terminus amino group is free. As shown in Figure 3A for $ppPLA_2$, the half-time for inactivation does not change when G:E > 2, and the residual activity of the modified enzyme was less than 10%. Comparable results were obtained with bovine AMPA.

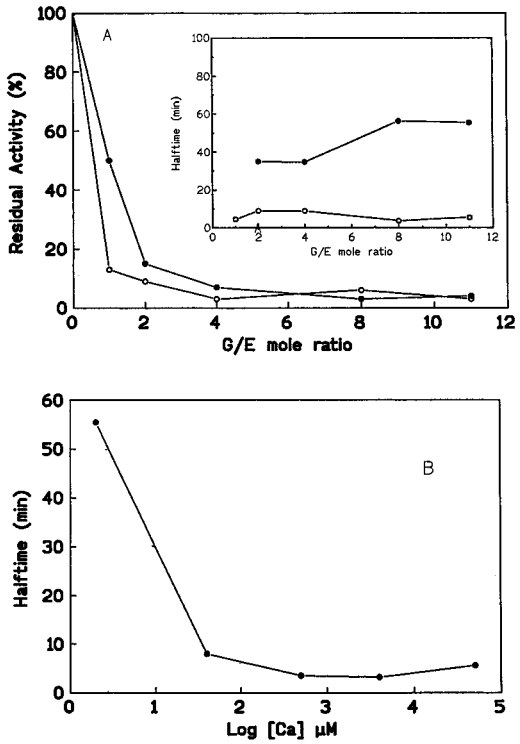


FIGURE 3: (A) Dependence of the residual activity after inactivation of ppPLA2 or (inset) of the corresponding half-times as a function of the gossypol:ppPLA2 (G:E) mole ratio in the presence of 0.5 mM calcium (open circles) or in the absence of calcium (closed circles). (B) Dependence of the half-time for inactivation of ppPLA2 by gossypol (G:E = 11) as a function of the calcium concentration. Other conditions were as in Figure 2.

The rate of inactivation is more rapid in the presence of calcium (Figures 2 and 3B). The apparent affinity for the effect of calcium on the inactivation half-time is about 0.06 mM (Figure 3B), compared to the dissociation constant of 0.35 mM for the enzyme in the aqueous phase or 0.25 mM at the interface (Jain et al., 1991a; Yu et al., 1993). These results show that the affinity for calcium increases 5−7-fold in the presence of gossypol. The converse is also true; i.e., enhanced binding of gossypol to PLA2 is seen in the presence of calcium. For example, gossypol quenches the Trp-3 fluorescence emission intensity of ppPLA2 immediately after mixing, and the intensity does not change after that. According to the two-step mechanism (eq 2), instantaneous quenching of Trp-3 by gossypol is due to the noncovalent binding, which precedes slower covalent inactivation (Figure 2). The emission intensity after the rapid noncovalent binding of gossypol depends on its concentration (Figure 4). A high affinity for the noncovalent binding of gossypol to PLA2 is indicated by the fact that the gossypol concentration used for these measurements is comparable to that of PLA2. Also the apparent affinity depends on the presence of calcium. For example, in a reaction mixture containing 2.6 μM ppPLA2, 50% quenching is seen at about 4 μM gossypol in the presence of calcium, and at about 18 μM in EGTA. Note that the quenching efficiency of gossypol is virtually the same for the ECa form as it is for the E*Ca·P form (Figure 4). Similar results were obtained with DTPM, the nonhydrolyzable ether analog of the substrate. Note that E*CaL forms of PLA2 are not covalently modified by gossypol, although both bind gossypol and are modified in the aqueous phase.

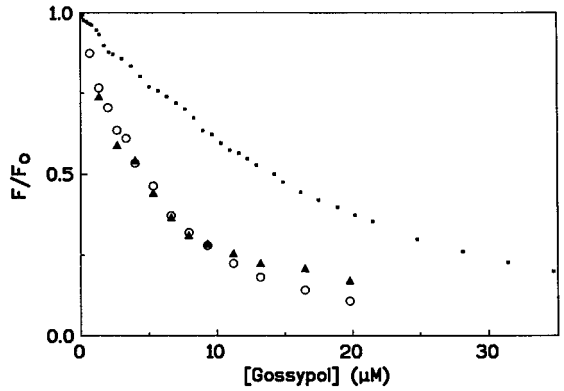


FIGURE 4: Relative fluorescence emission intensity of ppPLA2 (2.6 μM) immediately (5 s) after the addition of gossypol. The reaction mixture contained 10 mM Tris at pH 8.0 (dots) in the presence of 1 mM EGTA (E form) or (open circles) 0.5 mM $CaCl_2$ (ECa form) alone or with (closed triangles) 0.4 mM DMPM (E*CaP form).

Inactivation of the amino group at the N terminus of ppPLA2 by gossypol is a two-step process (eq 2) in which the initial rapid noncovalent binding is followed by Schiff base formation with the amino group of the N terminus with a half-time of about 5 min in the presence of calcium (Tables 1 and 2). Results in Figures 2−4 show that only the E or ECa form of the enzyme in the aqueous phase is covalently modified by gossypol, and the E* or E*L forms with or without calcium (where L = P, S, or I) are not modified by gossypol. Note that modification of additional amino groups, with a half-time of >20 min, may occur at high G:E ratios, which we have not characterized. The rapid noncovalent binding is indicated by the observation that the kinetics of modification are not limited by the concentration of free gossypol to the enzyme, as well as by the rapid quenching. The fact that the slower Schiff base formation step is facilitated by calcium suggests that a significant change at the N terminus is induced by calcium, which enhances the apparent affinity of gossypol. This is an intriguing observation, not only because the kinetics of gossypol modification are not influenced by the occupancy of the active site of the enzyme in the aqueous phase but also because, as also shown below, inactivation by gossypol is completely blocked if the enzyme is bound to the interface. These results suggest a complex interplay of catalytic and interfacial binding events through calcium, as also suggested by detailed kinetic analysis (Yu et al., 1993; Jain et al., 1993b) and site-directed mutagenesis (Huang et al., 1996).

Inactivation of ppPLA2 by Gossypol Occurs through Schiff Base Formation with α-NH_2 of Ala-1. Evidence that gossypol interacts with the amino group at the N terminus and forms a Schiff base, E=G in eq 2, is based on the following observations. (a) Peramidinated PLA2, AMPA, which has no other free amino group except the α-amino of Ala-1, is inactivated by a single time-dependent process (Figure 2). (b) AMPA is maximally inactivated by less than 2 equiv of gossypol (cf. Figure 3A). (c) The reaction product of ppPLA2 or AMPA with gossypol is stabilized after reduction with borohydride (Experimental Procedures). (d) The single free amino group in AMPA, which is modified by dansyl chloride, is not free in AMPA treated with gossypol. (e) The time-dependent loss of activity of ppPLA2 is not seen with gossypol pretreated with borohydride. (f) Localization of the gossypol moiety in the vicinity of the Trp-3 residue of ppPLA2 is also indicated by the fact that the emission intensity at 333 nm from the binding and

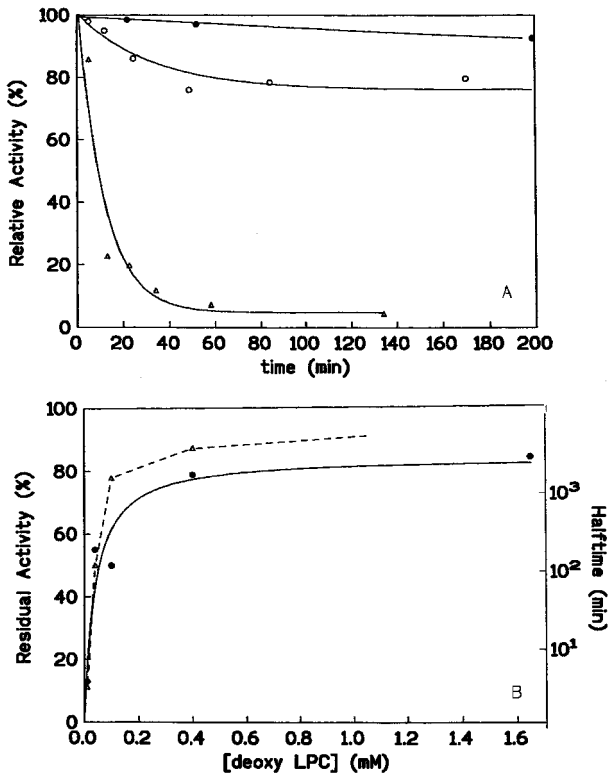


FIGURE 5: (A) Time course for inactivation by gossypol of ppPLA2 by gossypol in the (from the top down) E*I form (E + 0.4 mM deoxy-LPC + 8 μM MJ33; closed circles), E* form (E + deoxy-LPC; open circles), and EL form (E + MJ33; open triangles). (B) Residual activity (circles) and inactivation half-times (triangles) for ppPLA2 by gossypol (G:E = 11) as a function of deoxy-LPC concentration. In both cases, other conditions were as in Figure 2 with 0.5 mM Ca.

modification of ppPLA2 is reduced by 50% on modification by gossypol, and the intensity does not change appreciably on the denaturation of the enzyme under reducing conditions in dodecyl sulfate micelles or in an 8 M urea solution. (g) In addition, modification of ppPLA2 or AMPA by gossypol is accompanied by spectroscopic changes consistent with the formation of a Schiff base. The time-dependent increase in the absorbance at 400 nm is seen during the incubation of AMPA with gossypol, and the half-time for the absorbance change was the same as that measured by the assay of the residual activity (cf. Figure 2). The value of the molar extinction coefficient for E=G at 400 nm, compared with that of a reference standard prepared with phenylalanine, suggested that only one gossypol moiety is incorporated per 14 000 Da of ppPLA2 (AMPA). The absorption maximum at 400 nm disappears on reduction of E=G with borohydride, and the product is stable under HPLC conditions (Experimental Procedures). Although dialdehydes can form morpholino derivatives with a single amino group (King & Colman, 1983), it is unlikely with gossypol due to the constraints of the ring size and geometry.

Gossypol Does Not Inactivate PLA2 at the Interface. As shown in Figure 5A, the time course of modification of ppPLA2 by gossypol changes dramatically in the presence of deoxy-LPC, a neutral diluent (ND). Recall that ppPLA2 binds to aqueous dispersions of deoxy-LPC, and the active site of the bound enzyme remains unoccupied in the absence of added mimics (Jain et al., 1991b). This permits dissection of two sequential equilibria:

$$E + ND \rightleftarrows E^* + L \rightleftarrows E^*L \qquad (4)$$

At the ND interface, PLA2 is present only in the E* form, and both E and E* forms are alkylated by PNBr (Jain et al., 1991b). If a mimic (L = S, P, or I) is also present in the interface, the equilibrium shifts toward the E*L form, which is not alkylated by PNBr.

Results in Figure 5A show that only the E form is modified by gossypol and that E* and E*L forms are not. Results summarized in Figure 5B show that the inactivation time and the residual activity increase with increasing concentrations of deoxy-LPC, and virtually complete protection is seen at saturating concentrations above 0.15 mM. The initial steep dependence of the residual activity on deoxy-LPC concentration extrapolated to the maximum residual activity suggests that, at subsaturating concentrations of deoxy-LPC, PLA2 forms a stoichiometric complex with the diluent and only excess enzyme remains free to be modified by gossypol. These results are apparently surprising, yet consistent with eq 4. Our earlier results showed that the affinity of PLA2 for deoxy-LPC in the absence of an active site-directed mimic is rather poor (Jain et al., 1991a,b, 1995), and the dissociation constant for ppPLA2 from the interface decreases by 40−60-fold, from 1.6 to 0.02 mM, in the presence of an active site-directed substrate mimic (Jain et al., 1993b). Gel permeation studies on TSK-4000 also showed that the PLA2/ND mixture eluted as a high-molecular mass species only in the presence of gossypol or MJ33. In short, gel permeation studies and the inactivation kinetics studies suggest that the binding behavior of the E·G complex of ppPLA2 for the interface is comparable to that of the EL complex; however, results described next show that, unlike MJ33, gossypol does not bind to the active site of PLA2.

Results in Figure 5 and Table 2 show that the E* or E*L form of PLA2 is not modified by gossypol, while virtually no protection is seen in the EL form. Results in Table 2 also show that catalytic His-48 in the E or E* form is readily alkylated by PNBr, but the E·Ca, E*Ca, E*CaL, and ECaL forms are not alkylated [see Yu et al. (1993)]. Results in rows 1−6 show that gossypol modifies the E and ECa forms but not the E* or E*Ca forms. On the other hand, the half-time for the alkylation of His-48 is virtually the same in the E or E* form, and it is at least 50-fold higher for the ECa, ECaL, E*Ca, and E*CaL forms (Jain et al., 1991a; Yu et al., 1993). Protection from gossypol is not observed in the ECaL form for mimics (rows 7−9, Table 2) such as DC_8-PC, MJ33, or MJ72 present as solitary monomers in the incubation mixture below their critical micelle concentration. As expected, the half-time for alkylation by PNBr was modified under these conditions due to the occupancy of the active site. Particularly striking are the results in rows 10−12, where the half-time for inactivation by gossypol increases significantly, even though both DC_8PC-ether and inhibitors are present below their cmc, which provides an independent confirmation for the formation of E*L, also seen with spectroscopic and kinetic methods (Rogers et al., 1992, 1996).

To recapitulate, E or EL forms of ppPLA2 are inactivated by gossypol. E* and E*L forms are not, even though gossypol binds to these forms. Since gossypol modifies the α-amino group of Ala-1, it may be concluded that in E* and E*L forms the amino group becomes inaccessible for the modification. Although the kinetics of modification depend on the presence of calcium, the independence of the gossypol modification from events of the catalytic site is shown by the observation that the kinetics are not influenced by the occupancy of the active site, as in the EL form of the enzyme in the aqueous phase. Also, the gossypol-modified enzyme retains 2−10% of the residual activity. Next, we use gossypol to probe the basis for the kinetic effects of sulfated glycoconjugates and to resolve certain mechanistic alternatives, implied in eq 3, that can lower the observed rate of hydrolysis by secreted PLA2 under certain conditions.

Polyanionic Sulfated Glycoconjugates Reduce the Rate of Hydrolysis of DC_7PC Micelles by PLA2. The rationale for a detailed analysis of the effect of heparin and related conjugates on PLA2-catalyzed hydrolysis comes from a variety of observations. PLA2s retained on an immobilized heparin−agarose column are eluted with 0.1−1 M NaCl (Diccianni et al., 1990, 1991a). A role for electrostatic interactions during interfacial catalysis is indicated by the fact that anionic charge at the substrate interface promotes binding and hydrolysis by PLA2 (Jain et al., 1982; Ramirez & Jain, 1991). Thus, lower rates in the presence of heparin are attributed not only to reduced binding of PLA2 to the interface (Peers et al., 1987) but also to competitive inhibition (Dua & Cho, 1994) and allosteric modulation via the N terminus (Diccianni et al., 1991b). Indeed, there are several possible ways in which the rate of interfacial catalysis can be reversibly lowered (Jain & Jahagirdar, 1985; Gelb et al., 1994), and traditional assays used for earlier studies do not distinguish such possibilities. The mechanistic basis (eq 3) of the kinetic effects of heparin and proteoglycans, hsPG-I and -II from human aorta, is resolved below.

The rate of hydrolysis of micellar DC_7PC by hiPLA2 decreases in the presence of several sulfated glycoconjugates (Figure 6A). An about 95% loss of activity is seen with hsPG-I and -II, and in both cases, a 50% reduction in the rate is seen at about 15 μg in 4 mL of reaction mixture. The percents of reduction in the observed rate with 5 μg of hsPG-I for the hydrolysis of DC_7PC by PLA2 from the following sources are as follows: hiPLA2 (25%), ppPLA2 (20%), venoms of bee (34%), *Naja melanoleuca* DE2 (33%), the *Agkistrodon halis blomhoffii* acidic (55%) and basic enzymes (10%), *Hemachatus hemachatus* (10%), *Vipra rusellii* (11%), *Cortalus adamenteus* (22%), and *Notachus scrutatum* (11%). These results show that the hydrolysis by all the three evolutionarily divergent classes of PLA2 is influenced by hsPG-I.

The concentration dependence of HMW-heparin on the rate of hydrolysis of DC_7PC shows two steps (Figure 6A); about 40% of the activity is lost at 20 μg of HMW-heparin, and then a less pronounced effect is seen at higher concentrations. All commercially available heparin showed only a modest effect on the ppPLA2 or hiPLA2, comparable to that seen with chondroitin sulfate (Figure 6A); i.e., the initial sharp decrease is not seen. In fact, a modest increase in the rate was seen at low concentrations of some preparations of heparin [see also Sartipy et al. (1996) and a personal communication of M. H. Gelb]. While it is tempting to interpret the lower rates as a case of simple "competitive inhibition" (Dua & Cho, 1994), detailed analysis of the kinetic effects developed below shows that the underlying process is far more complex. In terms of eq 3, the apparent loss of activity is due to the formation of a complex of PLA2 with the conjugate in the aqueous phase, EH and EH_n, which can bind only an exchangeable substrate, such as DC_7PC, to support hydrolysis. An interfacial complex, E*H, is possibly formed with heparin added to the enzyme at the interface; however, it is catalytically functional. As shown

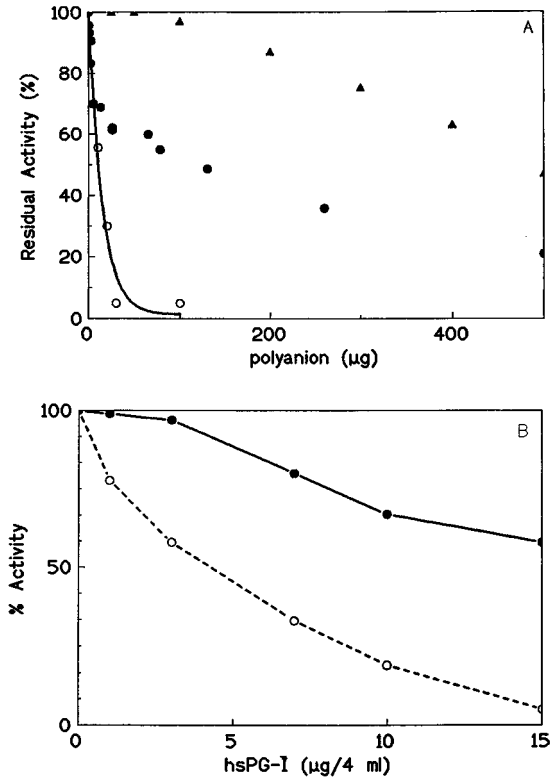


FIGURE 6: (A) Residual activity of hiPLA2 (36 pmol) on 3.1 mM DC_7PC in the presence of increasing amounts of (open circles) hsPG-II, (closed circles) HMW heparin, or (triangles) chondroitin sulfate A. (B) Residual activity of ppPLA2 (3 pmol) on 0.3 mM DC_8PM as a function of hsPG-II with 1 mM (open circles) or 500 mM NaCl (closed circles). In both cases, reaction progress was monitored by pH-stat titration at pH 8.0 and 24 °C in 4 mL of 0.5 mM $CaCl_2$ and 1 or 500 mM NaCl. Typically, the reaction was initiated by the addition of the enzyme; however, the order of addition of the components showed no significant difference in these two assays.

below, a dramatic loss of activity and other anomalous kinetic effects are associated with the formation of a high-molecular mass complex in the aqueous phase, EH_n, which does not interact with the substrate interface.

hsPG-I Has a Modest Effect on Interfacial Catalysis by ppPLA2 in the Scooting Mode. The rate of hydrolysis of anionic DC_8PM is lower in the presence of hsPG-I, and the effect is less pronounced in the presence of 0.5 M NaCl (Figure 6B). Not only is the effect of the conjugate and salt comparable to that seen with DC_7PC micelles, but the order of addition of components had little effect with micelles of DC_7PC or DC_8PM (not shown). In accord with eq 3, activity is determined by the fraction of enzyme in E* or E*H forms, whereas the loss of activity is putatively due to the enzyme sequestered as EH_n.

Results with short chain substrates do not distinguish between the kinetic effects due to perturbed binding of the enzyme to the interface, lower intrinsic activity of the E*H form, or trapping of the enzyme in the EH_n form. This analysis is possible with the scooting mode kinetics, where the order of addition of the components also becomes critical. For example, ppPLA2 remains bound to DMPM vesicles during the course of the reaction progress in the scooting mode (Jain et al., 1986, 1995). Thus, an apparent first-order reaction progress (Figure 7, curve a) results because the reaction ceases as the substrate on the enzyme-containing vesicle surface is exhausted. hsPG-I has a small but significant effect on the first-order course of the reaction

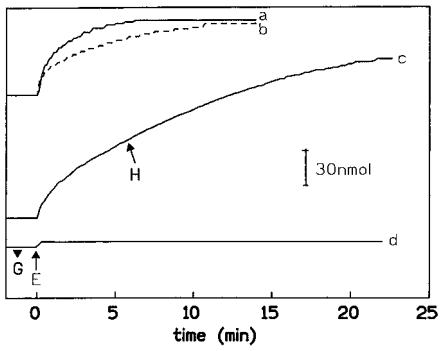


FIGURE 7: Reaction progress curves for the hydrolysis of 0.24 mM DMPM as sonicated vesicles in 4 mL of 0.5 mM $CaCl_2$ and 1 mM NaCl at pH 8.0 and 24 °C. The reaction was initiated with 14 pmol of ppPLA2 for curves a and b or 36 pmol of V3W hiPLA2 for curves c and d. The reaction mixture for curves b and d also contained 2.5 μg of hsPG-I. Note that in the scooting mode the extent of hydrolysis (curve a versus c) depends on the amount of enzyme.

progress by ppPLA2 (Figure 7, curves a and b). The half-time increases modestly without a significant effect on the extent of hydrolysis; i.e., hsPG-I does not promote the desorption of bound ppPLA2, nor does it promote fusion or lipid mixing between vesicles. If this were not the case, additional hydrolysis would be seen under these conditions [e.g., see Jain et al. (1991c)]. In short, hsPG-I has only a modest effect on the interfacial catalytic turnover at the interface; i.e., the activity of E*H is only modestly lower. Also, heparin did not show a significant effect on the scooting mode kinetics.

A dramatic effect of the order of addition of hsPG-I on the hydrolysis of DMPM vesicles is shown in Figure 7 (curves c and d). Although comparable results were obtained with ppPLA2, for comparative purposes, these studies were carried out with the V3W mutant of hiPLA2, which is also used later for the fluorescence binding measurements. For example, if 2.5 μg of hsPG-I is added to the reaction mixture before the enzyme, virtually no hydrolysis occurs. In contrast, only a modest decrease in the rate is seen if hsPG-I is added after the initiation of the reaction, that is when the enzyme is already bound to the interface. Results with a different sequence of additions (not described here) were always such that little hydrolysis is seen if the enzyme comes in contact with hsPG-I before it comes in contact with the stable DMPM interface; i.e., DMPM vesicles are not accessible to the EH_n complex in the aqueous phase. Thus, a large decrease in the observed rate is expected under conditions where the EH_n complex is preformed in the aqueous phase. This will have a significant effect on the rate if the enzyme returns to the aqueous phase during the course of reaction progress, as is the case with hiPLA2 (Bayburt et al., 1993) but not with its V3W mutant which binds more tightly to the interface (Othman et al., 1993). The stability of EH_n complexes of sulfated glycoconjugates depends on the nature of the enzyme and the salt concentration (see below).

Sulfated Glycoconjugates Do Not Influence the Binding of Substrate Mimics to the Active Site of PLA2. The half-time for alkylation of catalytic His-48 of PLA2 is very sensitive to the occupancy of the catalytic site (Jain et al., 1991a; Bayburt et al., 1993). Not only is the effect of heparin on the intrinsic catalytic turnover in the interface modest at best, as in the preceding subsection, but independent measurements also showed that K_{Ca}, K_{Ca}*, and K_P* values

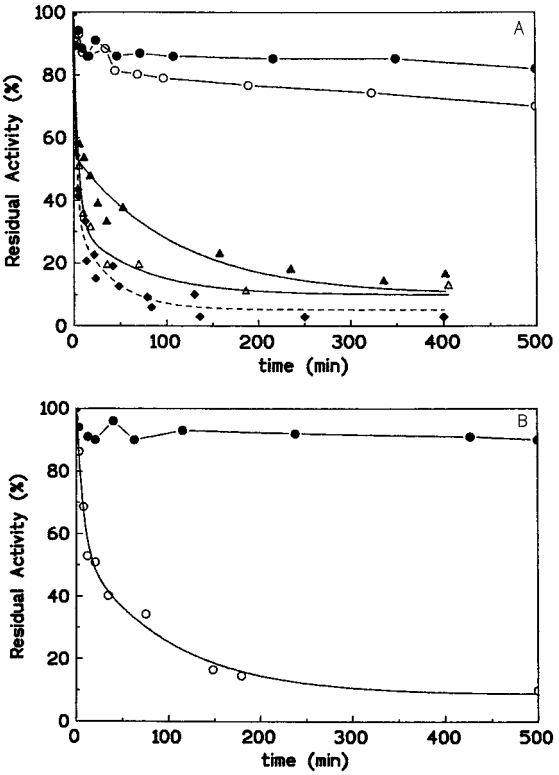


FIGURE 8: (A) Time course of inactivation by gossypol of the E* form of ppPLA2 in deoxy-LPC (closed circles) without or (open circles) with 10 μg of hsPG-I. The time course for the inactivation of the E form in the aqueous phase in the presence of (open triangles) 130 μg of heparin or (closed triangles) 10 μg of hsPG-I or (diamonds) without any additive. (B) Time course of inactivation of hiPLA2 by gossypol in the presence of 10 μg of hsPG-I for (open circles) the E or (closed circles) the E* form in the presence of 5 mM hexadecylphosphocholine (a neutral diluent for hiPLA2). The enzyme:gossypol mole ratio for these measurements was 11. Other conditions were as in Figure 2.

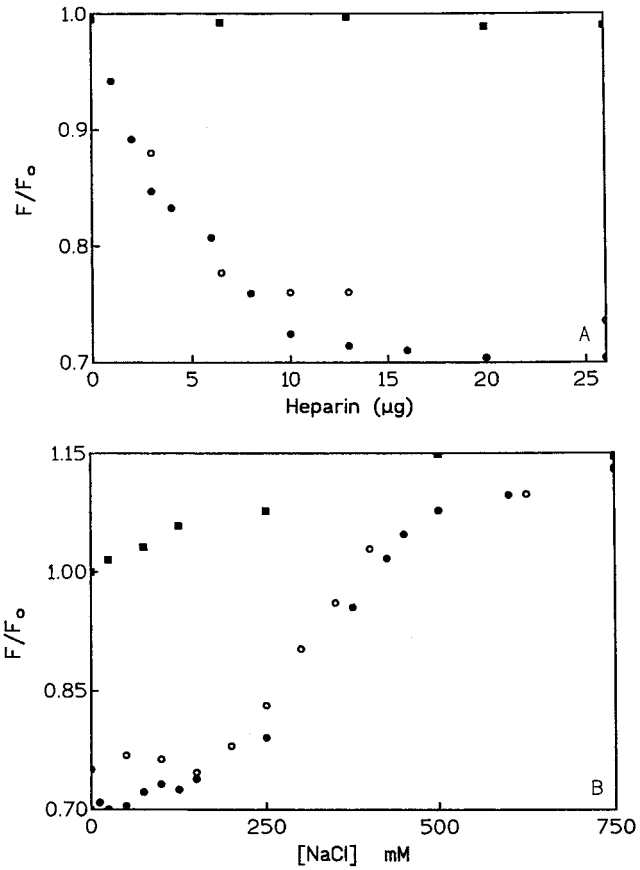


FIGURE 9: (A) Change in the fluorescence emission intensity of V3W hiPLA2 at 345 nm (excitation at 280 nm) as a function of heparin (reaction volume of 1.4 mL) in 10 mM Tris at pH 8.0: (closed circles) high-molecular mass heparin or (open circles) LMW-heparin and (squares) LMW-heparin in the presence of 0.8 M NaCl. (B) Salt concentration dependence of the change in the fluorescence emission from V3W hiPLA2 (square) alone or in the presence of 130 μg per 1.4 mL (open circles) high-molecular mass heparin or (closed circles) LMW-heparin.

for ppPLA2 or hiPLA2 did not change in the presence of heparin or hsPG-I. Also, calcium-dependent K_I* values for competitive inhibitors, MJ33 with ppPLA2 (Jain et al., 1991b) or HK40 with hiPLA2 (Bayburt et al., 1993), are not altered in the presence of the conjugates. These results show that the binding of conjugates must occur at a site other than the catalytic site, that the conjugate does not influence the binding to the catalytic site, and that the conjugate must not influence the binding to the catalytic site.

Heparin Does Not Influence the Accessibility of the α*-Amino Group.* hsPG-I or heparin has little effect on the rate of inactivation by gossypol. For example, ppPLA2 bound to deoxy-LPC is not inactivated by gossypol in the presence or absence of hsPG-I (Figure 8A). The time course of inactivation of the E form is not significantly influenced in the presence of heparin or hsPG-I. Similar results were obtained with hiPLA2 (Figure 8B). These results show that hsPG-I has only a modest effect on the time course of inactivation of ppPLA2 or hiPLA2 in the aqueous phase. The fact that the E* form of both of these enzymes is not inactivated in the presence of hsPG-I shows that the bound enzyme does not leave the interface in the presence of the glycoconjugate. The conclusion, from the kinetic results described in this and the preceding sections, that the conjugates bind to a site other than the catalytic site or the i-face of PLA2 is also supported by spectroscopic results.

Spectroscopic Evidence for the Binding of Heparin to the E Form. Results in Figure 9A show that heparin quenches the fluorescence emission from Trp-3 of the V3W mutant of hiPLA2, and that there is little effect of heparin in the presence of 0.8 M NaCl. Qualitatively similar results were obtained with ppPLA2 and isoPLA2 (Table 3). Although the heparin-induced decrease in the Trp-3 emission was about 30% in all cases, the concentration of heparin required for half of the change, the I_{50}(heparin) values in Table 3, is appreciably different for the three PLA2s. Although the structural basis for the quenching is not known, the results described below suggest that it is due to the formation of EH_n aggregates. For example, the effect of the NaCl concentration on the fluorescence emission of V3W hiPLA2 with or without heparin, shown in Figure 9B, clearly shows that the effect of heparin on the environment of Trp-3 is completely reversed by NaCl. I_{50}(NaCl) values (Table 3), the salt concentrations required for a 50% decrease of the effect at the saturating heparin concentration, suggest that the EH_n complex of V3W hiPLA2 is appreciably more stable.

The accessibility of Trp-3 in the complex of PLA2 with heparin was monitored by fluorescence quenching by acrylamide, a water-soluble quencher. The Stern−Volmer plots, from which the K_{SV} values were obtained, suggest a collisional quenching mechanism. Stern−Volmer constants (K_{SV}) in Table 3 suggest that Trp-3 in the E form can be accessed from the aqueous phase (Jain & Maliwal, 1993). The K_{SV} value decreases significantly in the presence of heparin or hsPG-I, and the decrease is lost at 0.5 M NaCl

Table 5: Elution Times[a] (Minutes) for PLA2s from a TSK-250 HPLC Column

	alone		with heparin (1:15)	
PLA2	no NaCl	0.48 M NaCl	no NaCl	0.48 M NaCl
ppPLA2	10.2	10.3	5.4	10.5
iso-ppPLA2	9.9	9.9	5.6	9.9
pro-ppPLA2	9.9	9.9	5.7	9.9
bovine PLA2	10.3	10.3	5.6	10.3
V3W hiPLA2	9.6	11.2	4.2	11.2

[a] Conditions as in the footnote to Table 4 with details in Experimental Procedures.

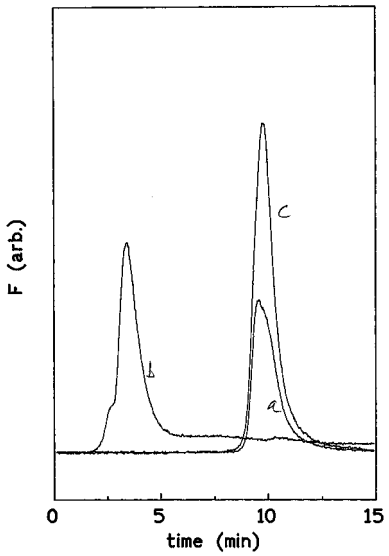


FIGURE 10: HPLC elution profiles from a TSK-250 gel permeation column monitored as the tryptophan fluorescence signal (a) for 8 μg of ppPLA2 alone, (b) with 0.12 mg of 3 kDa heparin with 10 mM NaCl, or (c) with 0.48 M NaCl in the elution buffer. Other conditions are given in Experimental Procedures. The flow rate was 1 mL/min.

(not shown), which is consistent with the salt-induced dissociation of the complex. Note that the accessibility of Trp-3 in the EH_n form is lower compared to that of the E form; however, the shielding is far more pronounced in the E*L form on DTPM.

Gel Permeation Behavior of EH_n Complexes. Complexation of ppPLA2 with heparin was characterized by gel permeation through a TSK-250 column (Tables 4 and 5), which adequately separated 14 kDa PLA2 from larger complexes (Figure 10). The proportion of enzyme eluting as a high-molecular mass complex increases with the ratio of heparin to ppPLA2. The elution profile also shifts toward the higher-molecular mass range, suggesting that a range of complexes are formed. As summarized in Table 4, at the flow rate of 1 mL/min, all four samples of heparin eluted at 5 min (compared to the void volume of 3.9 min). On the other hand, in the presence of 0.48 M NaCl, heparins elute as lower-molecular mass species with elution times of 7−10 min. Results in Table 4 also show that at low salt ppPLA2 eluted with the aggregated heparin peak, but at 0.48 M NaCl heparin and PLA2, peaks correspond to the lower-molecular mass species. The elution behavior of the four heparin preparations as complexes with ppPLA2 was comparable with significant quantitative differences. The effect of salt concentrations on the elution profile indicated that the proportion of ppPLA2 that eluted with the higher-molecular mass complex decreases with [NaCl]. We did not investigate these complexes in detail, not only because heparins and hsPG are microscopically heterogeneous but also because the tryptophan fluorescence from the protein component of hsPG, and a lack of tryptophan in the synovial PLA2, make it more difficult to do the kind of analysis which have been carried out with heparins and ppPLA2. At least qualitatively, these results clearly show that the heparin−ppPLA2 complexes exist as large molecular mass species, the EH_n form in the absence of NaCl.

Comparable elution behavior is seen with all the five PLA2s (Table 5), indicating that they form a stable complex with heparin which dissociates with added NaCl. This similarity, despite significant differences in their interface binding behavior, suggests that the primary effect of salt is on the stability of EH and EH_n complexes. Note that pro-ppPLA2 with seven extra amino acid residues at the N terminus behaves just like ppPLA2, which also rules out a role for Ala-1 in heparin binding.

Heparin Binds to PLA2 at the Interface. Measured as an increase in the fluorescence emission from Trp-3, the high-affinity binding of V3W hiPLA2 to anionic vesicles (Figure 11A) is similar to that seen with pancreatic enzymes (Jain & Vaz, 1987). Also as shown elsewhere (Othman et al., 1996), compared to the wild type, the V3W mutant has a somewhat higher affinity for the interface, as is the case with the W3 mutants of pancreatic PLA2 (Liu et al., 1995). Only the magnitude of the change depends on the source of the enzyme; however, a linear increase with the bulk lipid concentration shows that apparent K_d, the dissociation constant for the bound enzyme, is much smaller than 50 μM. The initial region of this curve extrapolated to the maximum change suggests that each enzyme molecule binds to about 25 molecules of DMPM at the interface.

It is particularly striking that the fluorescence change, associated with the binding of PLA2 to the interface, is not seen on the addition of DMPM if heparin or hsPG-I is present in the reaction mixture (Figure 11A). Similar behavior (Figure 11B) was seen if the binding of V3W hiPLA2 to the interface was monitored by resonance energy transfer from Trp-3 to the dansyl fluorophore, DNS-DTPE, at the interface (Figure 11B). The increase in the energy transfer intensity at 500 nm (excitation at 292 nm) is seen with increasing enzyme concentrations, and a 3-fold increase is seen at saturating levels of the enzyme where the vesicle surface is completely covered by the enzyme. In the presence of heparin in the reaction mixture, the change in the resonance energy transfer intensity is considerably smaller and the increase is seen only at higher enzyme concentrations, where only the enzyme not bound to heparin is available for the binding to the interface. Results in panels A and B of Figure 11 show that the enzyme bound to heparin (as the EH_n complex) cannot bind, nor does it dissociate for the binding of free enzyme, to DTPM vesicles. Note that addition of heparin or hsPG-I to the E*L (DMPM) form on vesicles does not cause a significant desorption of the bound enzyme. If the enzyme was desorbed, a decrease in the emission from Trp-3 or the energy transfer signal will be seen. In fact, as shown in Figure 11C, addition of heparin causes a small (12%) but significant increase in the energy transfer intensity and a small (4%) decrease in the Trp-3 emission. hsPG-I also showed a similar behavior (results not shown). Collectively, spectroscopic results confirm the kinetic results and show that the conjugates bind to PLA2 at the interface without dislodging it.

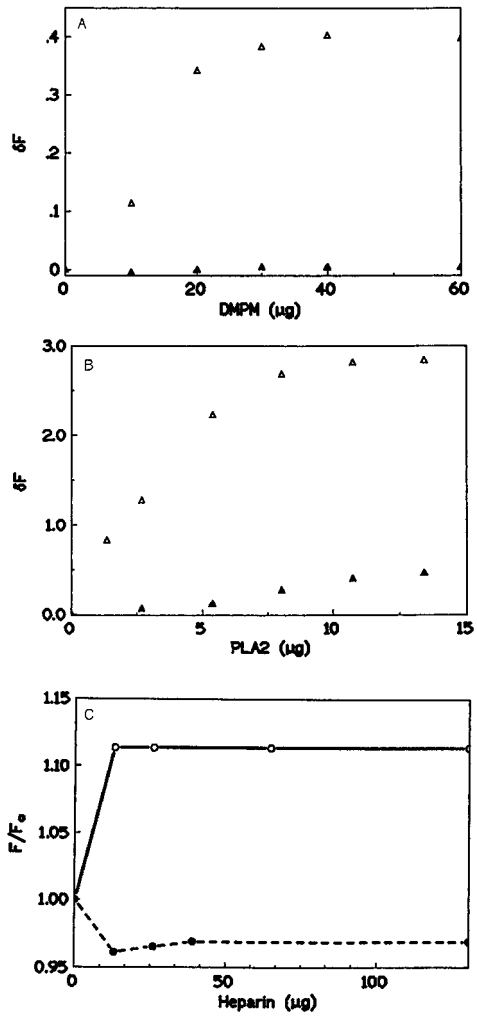


FIGURE 11: (A) Change in the fluorescence emission intensity at 333 nm (excitation at 292 nm) of V3W hiPLA2 (40 μg) as a function of DMPM concentration in the (open triangles) absence or (closed triangle) presence of 130 μg of heparin (reaction volume of 1.4 mL). Similar results were obtained in the presence of hsPG-I instead of heparin. (B) Change in the resonance energy transfer intensity at 500 nm (excitation at 292 nm) on the addition of V3W hiPLA2 to 2% dansyl-DTPE in DTPM covesicles in the (open triangles) absence or (closed triangles) presence of 130 μg of heparin. Similar results were obtained in the presence of hsPG-I instead of heparin. (C) Effect of heparin on (closed circles) the intrinsic Trp-3 fluorescence emission of V3W hiPLA2 bound to saturating DMPM as in panel A or (open circles) resonance energy transfer as in panel B of V3W hiPLA2 bound to dansyl-DTPE/DTPM covesicles.

DISCUSSION

The independence of the catalytic site from the interfacial binding region of PLA2 is a key feature of interfacial catalysis (Berg et al., 1991; Jain et al., 1995), and thus, the interfacial kinetic behavior of PLA2 is a functional measure of the enzyme at the interface (Jain & Berg, 1989). The fact that gossypol does not inactivate PLA2 at the interface makes it a useful probe for sorting out interfacial binding events associated with complex kinetic situations induced by glycoconjugates. Results described here show that PLA2 in the aqueous phase forms the EH_n complex which makes PLA2 inaccessible to the interface (eq 2). There are indications that the conjugates bind to PLA2 bound to the interface, the E* form, with a modest effect on the catalytic turnover; however, the conjugates do interfere with alkylation of catalytic residue His-48. Together, these results show that sulfated glycoconjugates do not directly interfere with the catalytic or interfacial binding behavior of PLA2. The significance of these observations is discussed below.

Drug Potential of Gossypol as an Inhibitor of PLA2. Male antifertility properties of cotton seed have been attributed to gossypol (National Coordinating Group on Male Antifertility Agents, 1978). It is a polyphenolic dialdehyde (Figure 1), which can form a Schiff base with amino groups. Therefore, it is not surprising that gossypol modulates activities of several enzymes (Olgiati et al., 1984; Ohuchi et al., 1988); however, modification of PLA2 with stoichiometric amounts of gossypol is probably the most dramatic of such effects. Evidence for potential PLA2 targets of gossypol is indicated by several reports. Gossypol modulates the acrosomal reaction (Yanagimachi et al., 1982). PLA2 activity is implicated in the acrosomal reaction (Llanos et al., 1982). A PLA2 has been isolated from human spermatozoa (Vainio et al., 1985). The tumor promoter-induced arachidonate metabolism in peritoneal macrophage, which secretes hiPLA2, is inhibited by gossypol (Ohuchi et al., 1988). It is also worth noting that gossypol inhibits cholesterol and triglyceride absorption (Shandilya & Clarkson, 1982), as do inhibitors of PLA2 (Homan & Krause, 1997). In short, some of the cellular functions modulated by gossypol could have their origin in the role of PLA2.

Gossypol Is a Probe for the α-Amino Group of Ala-1 of ppPLA2. Specific probes are valuable tools for resolving molecular events underlying more complex kinetic situations. As a probe for the interfacial recognition region (i-face) of PLA2 (eq 3), gossypol has several attractive features. The inactivation is specific for the α-amino group of Ala-1. It is virtually stoichiometric. The equilibrium for the Schiff base is reversed on the removal of the reagent. Gossypol modifies all three evolutionarily divergent classes of secreted PLA2s (Table 1). As a probe for PLA2, it complements the alkylating reagents for catalytic His-48 (Verheij et al., 1981). For example, phenacyl bromide, which is reasonably selective for His-48, must be used in stoichiometric excess; however, it has proven to be remarkably useful for determination of the binding equilibria for active site-directed substrate mimics (Jain et al., 1991a; Yu et al., 1993, 1997). Note that modification of PLA2 by manoalogue occurs at considerably higher than stoichiometric concentrations, and it also modifies several amino groups (Ghomashchi et al., 1991).

The usefulness of gossypol as a probe comes from the fact that its reaction with ppPLA2 and hiPLA2 is virtually completely blocked in the E* or E*L form, where L is a mimic bound to the active site. The key result reported here is the fact that complexation of PLA2 with heparin or hsPG-I does not significantly interfere with the catalytic or the interfacial recognition function of PLA2. The gossypol modification results also support the notion that the N terminus is a part of the i-face of PLA2 (Dijkstra et al., 1983; Ramirez & Jain, 1991). It is most surprising that binding of calcium to PLA2 makes it more susceptible to gossypol and the rate of inactivation is significantly faster in the presence of calcium (Figures 3 and 4). The X-ray structure of the Ca−PLA2 complex has been characterized (Dijkstra et al., 1983; Scott et al., 1991; Scott & Sigler, 1994), but the structure of the calcium-free enzyme is not available. Thus, distal structural changes associated with the binding of calcium to PLA2 are not established. However, note that a calcium-induced UV spectral change in the 290 nm region ppPLA2 has been reported (Verheij et al., 1981), presumably

due to the perturbation of Trp-3. Our results are also consistent with the observations that calcium is not required for the binding of PLA2 to the interface, that the binding of a ligand to the active site is not required for the binding of PLA2 to the interface, and that under certain conditions premicellar aggregates of PLA2 are formed with monomeric amphiphiles (Table 2).

Sequestering of PLA2 by Heparin. A key result reported here is the fact that secreted PLA2 forms a high-molecular mass complex (EH_n) with glycoconjugates, and that PLA2 sequestered in these complexes is not readily accessible to the substrate in the bilayer form. The fact that both the catalytic site and the i-face of PLA2 in EH_n complex are free and not perturbed is also suggested by the kinetic and protection studies. Evidence for an apparently high affinity of heparin binding to PLA2 comes from conditions that promote formation of EH_n. Although it is not a focus of the present study, formation of a stable high-molecular mass complex of PLA2 and heparin (Figure 11, Tables 4 and 5) must involve multiple interactions between several PLA2 and heparin molecules. It is however clear that the EH_n complex sequesters PLA2 and makes it inaccessible for the hydrolysis of nonexchangeable substrate on vesicles.

At this stage, one can only speculate about whether there is a heparin binding site on PLA2, which for the present discussion is called the h-site. Immunological evidence for an h-site on PLA2 has been reported (Murakami et al., 1991). Our results show that heparin interacts with PLA2 at the interface with little perturbation of the interfacial catalytic cycle, which indicates that the h-site is distinct from the active site with His-48, or from the i-face of PLA2 with Ala-1. The conjugates bind to E, E*, and E*L forms of PLA2, and that with hsPGs the E*H complex (Figures 6, 7, and 11C, Tables 4 and 5) of hiPLA2 has a modestly (50%) lower rate of interfacial catalytic turnover without desorption of the enzyme from the interface. In terms of eq 3, the primary evidence for the interactions involving the h-site comes from the E*H or EH species, and not from the EH_n complex. Although the EH_n complex is very stable in the absence of salt, the salt dependence shows that relatively weak ionic interactions are at work in these complexes. Since the salt concentration dependence is different for PLA2s from different sources, cationic groups on PLA2 exposed to the aqueous phase in the E* form of PLA2 must be involved in the putative h-site.

The structural rationale for electrostatic heparin−PLA2 interaction comes from the fact that heparin is a polyanionic linear copolymer of glycosamine and uronic acid with numerous sulfate ester linkages. hsPGs contain a characteristic core protein conjugated with sulfated glycosaminoglycan chains attached to a serine-glycine-X segment (Jackson et al., 1991). In addition to the possibility of bonding through multiple sites, complex structural and functional issues regarding the role of these conjugates are difficult to resolve because most glycoconjugate preparations are heterogeneous with respect to the peptides, sugars chains, and the degree of N and O derivatization of sugars by sulfates.

Possible Physiological Role for PLA2 Complexes of Heparin Conjugates. Sulfated glycoconjugates bind to a wide variety of proteins. This fact provides a strong rationale for the interaction of several plasma proteins with the hsPG type of molecules present on the subendothelial matrix. In analogy with the heparin binding region RLTRKRGLK or SVKAETKKQKHRH of apoB-100, it has been suggested that a cationic segment, such as KRLEKR (53−58) of PLA2, could be a part of the h-site on hiPLA2 (Dua & Cho, 1994; Kinkaid & Wilton, 1995). It appears that the role of specific residues in stabilizing heparin binding is rather modest, as expected for weak electrostatic interactions. For example, mutation of cationic residues in the heparin binding site of antithrombin results in only a modest (<3-fold) decrease in the binding (Meagher et al., 1996).

hsPGs are major components of the subendothelial layers of the arterial walls (Camejo et al., 1993). The observation that secreted PLA2 form the EH_n type of complex supports the view that the role of hsPG is to sequester circulating hydrolytic enzymes (Bosman et al., 1988), including lipoprotein lipase (Hultin et al., 1992; Lookene et al., 1996), hiPLA2 (Sartipy et al., 1996), and serum lipoproteins (Hurt-Camejo et al., 1990). Beyond the scavenger role, more specific functions for such interactions have also been described (Ross, 1993; Cheng et al., 1993; Suga et al., 1993), and the function of the peptidoglycan from the extracellular matrix of artery vessel wall is still being debated (Sartipy et al., 1996). Much of the discussion is now focused on the consequences of the view that in subendothelial matrix the proteoglycans retain and sequester cationic proteins from plasma, and therefore increase their residence time. For example, depending on the relative affinities of hiPLA2 for low-density lipoproteins and peptidoglycan, and the salt concentration in the extracellular matrix, increasing the residence time of hiPLA2 will lead to an increased production of bioactive lipids which trigger undesirable cell responses in the artery wall, including degeneration of macrophage into foam cell.

On the basis of our results, it can now be said that hiPLA2 bound to proteoglycans at the subendothelial surface would be catalytically functional with their active site exposed to the aqueous environment. Of course, the interfacial catalytic function of the bound enzyme will be expressed only if the substrate can access the i-face of the bound enzyme. In this context, the fact that LDL is also sequestered in the proteoglycan matrix deserves serious attention (Camejo et al., 1993, 1996). It is also relevant to point out that the 44 kDa calcium-independent PLA2 (PAF-hydrolase) present in plasma is neither inhibited nor sequestered by proteoglycans (R. Apitz-Castro and M. K. Jain, to be published).

To recapitulate, sulfated glycoconjugates sequester secreted 14 kDa PLA2 in a high-molecular mass stable complex, and such a complex could sequester the circulating PLA2. In the complex, catalytic site and interface recognition regions of PLA2 are exposed to the aqueous environment. Sequestration of PLA2 can have a significant role in regulating the formation of bioactive lipids produced by lipolysis, and changes in the proteoglycan matrix (related to age or disease) could result in the loss of this regulatory role.

ACKNOWLEDGMENT

We thank Dr. G. Camejo (Astra Hassle, Goteborg, Sweden) and Dr. Eva Hurt Camejo (Sahlgrenska University Hospital, Goteborg, Sweden) for bringing to our attention the problem of PLA2 interaction with heparin conjugates and for many useful suggestions. We also thank Professor Michael Gelb (University of Washington, Seattle) for comments on the draft manuscript.

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BI962972I