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Journal of Chromatography A, 731 (1996) 139–151

JOURNAL OF CHROMATOGRAPHY A

Affinity purification of phospholipase A_2 on immobilized artificial membranes containing and lacking the glycerol backbone

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First received 5 July 1995; revised manuscript received 20 September 1995; accepted 27 September 1995

Abstract

Immobilized artificial membranes (IAMs) are chromatography surfaces containing monolayers of phospholipid ligands. ^{ether}IAM.PC$^{C10/C3}$ contains the glycerol backbone whereas $^{\delta G}$IAM.PC$^{C10/C3}$ lacks the glycerol backbone. Affinity purification of PLA_2 on these IAM surfaces demonstrated that the surface structural differences were not important for phospholipase A_2 (PLA_2) binding. This suggests that the chromatographically important binding event involves the PLA_2 surface and the monolayer of polar choline headgroups on the IAM surface. After sample loading, short-chain alkylsulfonates were used as low eluotropic strength detergents to remove contaminating proteins, and PLA_2 were eluted with CH_3CN (30%). Octyllysophosphatidylcholine (0.5%) can replace CH_3CN to elute PLA_2 from IAM surfaces. The PLA_2 purity after IAM chromatography depends on the protein loading; analytical-scale loadings (0.8 mg protein/g IAM) resulted in a PLA_2 purity of ca. 70% based on densitometric scans of proteins in polyacrylamide gels after electrophoresis. Preparative loadings of 3.21 mg protein/g IAM resulted in 48% PLA_2 purity. Purification of PLA_2 to electrophoretic homogeneity was achieved using an IAM column followed by a strong anion-exchange column. These results suggests that IAMs may be used to develop purification methods for PLA_2 enzymes obtained from diverse biological specimens.

Keywords: Immobilized artificial membranes; Phospholipids; Phospholipase A_2; Enzymes

1. Introduction

IAM surfaces are immobilized membranes prepared by covalently binding phospholipids (PLs) to solid surfaces at a monolayer density. The synthesis [1,2] and characterization [3–5] of IAM surfaces have been described and several applications reported [6–11]. The application of IAMs for the purification of membrane proteins is slowly progressing [7–9,11,12] and the results suggest that IAMs may be a viable method for purifying several different membrane proteins. Recently, bovine pancreatic phospholipase A_2 (PLA_2) was purified to homogeneity in a single step using an ^{ester}IAM.PC$^{C10/C3}$ surface [12]. In that work, *Crotalus atrox* PLA_2 was purified to homogeneity using IAMs as the first chromatographic step and an anion-exchange column as the second step. It is interesting that bovine pancreatic PLA_2 is active as a monomer whereas

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0021-9673/96/$15.00
SSDI 0021-9673(95)01100-5

Crotalus atrox PLA_2 is active as a dimer [13], yet IAMs could be used to purify both enzymes.

A major objective of this work was to elucidate the surface recognition properties of PLA_2s for immobilized monolayers of PC ligands. Our goal was to determine if immobilized PC lipids required the glycerol backbone, ester groups or two alkyl chains to exhibit high affinity for PLA_2 enzymes. IAMs with and without the glycerol backbone and esters were prepared from PC ligands containing either one or two alkyl chains (Fig. 1). As shown in this paper, the glycerol backbone and ester linkages or the number of alkyl chains are not major lipid structural features required for high affinity between immobilized PC lipids and PLA_2 enzymes; this is most likely true for PLA_2 binding to fluid membrane surfaces.

2. Experimental

2.1. Chemicals and reagents

Reagents and molecular mass markers for gel electrophoresis were purchased from Bio-Rad (Hercules, CA, USA). Sodium butanesulfonate (C4-SO_3), sodium octanesulfonate (C8-SO_3) and sodium decanesulfonate (C10-SO_3) were obtained from Aldrich (Milwaukee, WI, USA), acetic acid, sodium phosphate, ethylene glycol (EG), silver nitrate, 37% formaldehyde solution and scintillation fluid (ScintVerse II) from Fisher Scientific (Pittsburgh, PA, USA), absolute ethanol from McCormick Distilling (Pekin, IL, USA), sodium carbonate, calcium chloride and sodium chloride from J.T. Baker (Philipsburg, NJ, USA) and glycine, sodium deoxycholate (DOC), trichloroacetic acid (TCA), trypsin Type I, Tris base, phosphate buffered saline (PBS) and PLA_2 from *Apis mellifera*, *Bungarus multicinctus* venom and *Crotalus atrox* PLA_2 from Sigma (St. Louis, MO, USA). Lyophilized *Crotalus atrox* venom was also obtained from Miami Serpentarium Labs. (Punta Gorda, FL, USA). BCA protein assay kits were purchased from Pierce (Rockford, IL, USA). [^{14}C]DPPC (112 mCi/mmol) was purchased from Amersham Life Science (Arlington Heights, IL, USA). DPPC and octyl lysophosphocholine were obtained from Avanti Polar Lipids (Alabaster, AL, USA).

2.2. IAM chromatography

^{ether}IAM.PC$^{C10/C3}$ and $^{\delta G}$IAM.PC$^{C10/C3}$ chromatography packing material were synthesized and columns were packed with this material either in our laboratories or at Regis Technologies (Skokie, IL, USA). Briefly, the preparation of these IAM surfaces involves preparing an ω-carboxyphosphatidylcholine (PC) ligand that is immobilized on silica propylamine at approximately a monolayer density [1,2,5,14]. After the PC ligand has been immobilized, residual amines are end-capped with decanoic anhydride (C10 groups) followed by propionic anhydride (C3 groups). Fig. 1 shows the general structures of the PC, C10 and C3 groups comprising the IAM surfaces used for this study.

The $^{\delta G}$IAM.PC$^{C10/C3}$ column is commercially available as an IAM.PC.DD column from Regis Technologies. IAMs were prepared from 12-μm aminopropylsilica containing 300-Å pores. Pilot columns of 3×0.46 cm I.D., analytical columns of 10 or 15×0.46 cm I.D. and semi-preparative columns of 6.4×1 cm I.D. were used. Pilot columns are short, inexpensive columns typically used for preliminary protein purification studies. However, regardless of the size of the IAM column used for protein purification, IAM guard columns were always used to increase the column lifetimes. Our Rainin Rabbit-HP HPLC system has been described [12]. All mobile phases were filtered through a 0.2-μm nylon 66 filter (Rainin). Mobile phase A contained 50 m*M* Tris–HCl (pH 7.2) and 25 m*M* KCl. Detergent solutions were prepared by dissolving their salts in mobile phase A and filtering using a syringe filtration device containing a Nalgene cellulose acetate 0.2-μm filter. In this work, the protein loading step utilized a 0.2 ml/min flow-rate until the mid-point of the "pass-through" peak. The flow-rate was then increased as shown in the chromatograms. The slow flow-rate during the loading step facilitated affinity interactions between the injected proteins and the IAM surface.

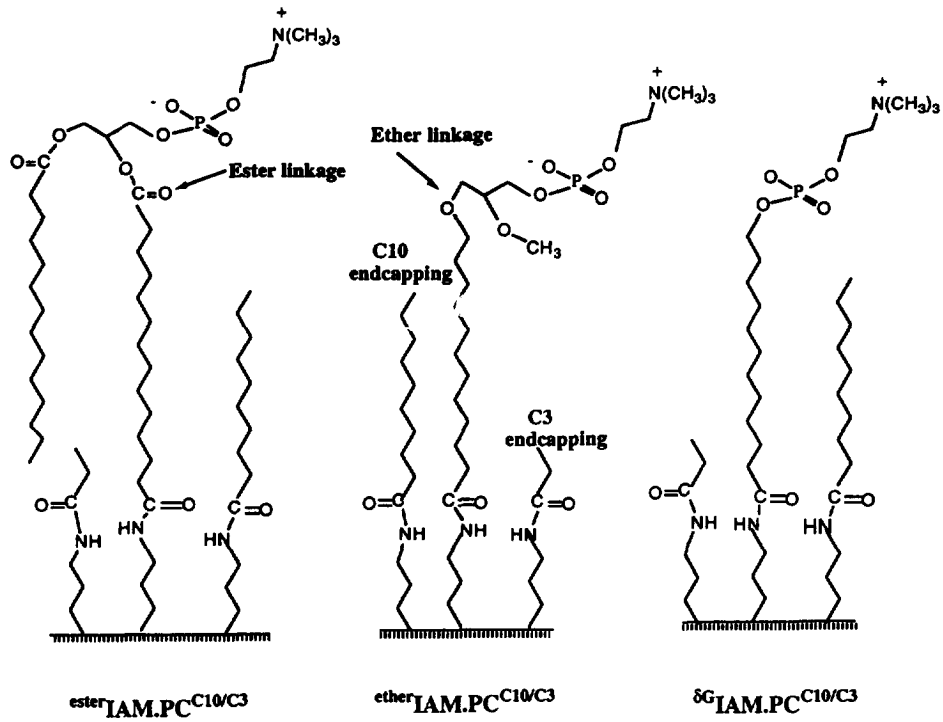

Fig. 1. General structures of IAM surfaces. ^{ester}IAM.PC$^{C10/C3}$ surfaces have PC ligands containing two acyl chains linked to the glycerol backbone by ester linkages. ^{ether}IAM.P PC$^{C10/C3}$ surfaces have PC ligands containing a single alkyl chain linked to the glycerol backbone through an ether linkage. $^{\delta G}$IAM.PC$^{C10/C3}$ surfaces have the glycerol backbone removed; an alkyl group is linked directly the PC group.

2.3. Anion-exchange chromatography

PLA_2 eluting from IAM columns is typically 50–70% pure depending on the protein loading and the size of the IAM column. Purification of PLA_2 to homogeneity thus requires a second chromatographic step. Anion-exchange columns have been used to purify PLA_2 [15,16] and therefore PLA_2 eluting from $^{\delta G}$IAM.PC$^{C10/C3}$ columns was purified to homogeneity using a Hydropore strong anion-exchange column (10 × 0.46 cm I.D.) containing 12-μm spherical particles and 300-Å pores; Rainin). Prior to injecting PLA_2 into the anion-exchange column, CH_3CN was removed by evaporation under a stream of nitrogen. The anion-exchange column was equilibrated with a solution prepared from 50 m*M* Tris–HCl (pH 6.2)–25 m*M* KCl. Pure *Crotalus atrox* PLA_2 eluted at approximately 0.200 *M* KCl.

2.4. Crotalus atrox PLA_2 sample preparations

A 100-mg amount of lyophilized *Crotalus atrox* venom powder was dissolved in 20 ml of PLA_2 sample buffer [25 m*M* $CaCl_2$–50 m*M* Tris (pH 7.6)] and filtered through a 0.2-μm Nalgene cellulose acetate filter before injection into IAM columns. The protein concentration of this solution was approximately 4 mg/ml.

2.5. SDS-PAGE

Fractions of 1 ml were routinely collected from IAM columns and analysed for proteins by SDS-PAGE analysis as described [12].

2.6. Scanning densitometry

Gels were scanned on a Shimadzu CS9000U dual-wavelength scanner using the transmission

mode. Silver-stained gels were scanned at 400 nm using a 0.05 × 2.0 mm beam size and 1 mm width for peak integration.

2.7. Protein content measurements

Protein contents in chromatographic fractions were measured using the bicinchoninic (BCA) protein assay kit (Pierce). Complete details are available [12].

2.8. Phospholipase A_2 assay

The procedures used to monitor PLA_2 activity in chromatographic fractions have been described [12].

3. Results and discussion

Linear alkyl sulfonates have been used to increase the eluotropic strength of mobile phases [17,18] and we have qualitatively classified protein affinity to IAMs based on protein elution using alkyl sulfonates in the mobile phase (Table 1). Short-chain alkyl sulfonate C4-SO_3 at a concentration of 1% (w/v) does not have a sufficient eluotropic strength to elute proteins from the ^{ester}IAM.PC$^{C10/C3}$ column, but 5–10% (w/v) C4-SO_3 elutes several proteins; thus, these proteins are referred to as low-affinity proteins (Table 1). High-affinity proteins such as pancreatic PLA_2 do not elute with C4-SO_3 even up to 30% (w/v). However, mobile phase concentrations either close to or above the CMC (2.1%, w/v) of the longer chain alkyl sulfonate C8-SO_3 can be used to elute pancreatic PLA_2 from the ^{ester}IAM.PC$^{C10/C3}$ column. Thus, the alkyl chain length of the alkyl sulfonate is clearly important for controlling the eluotropic strength of the mobile phase. C8-SO_3 (1%, w/v) is below its CMC (CMC = 2.1%) [19], but this concentration is still capable of eluting proteins from IAMs, although it will not desorb pancreatic PLA_2 from either the ^{ether}IAM.PC$^{C10/C3}$ or $^{\delta G}$IAM.PC$^{C10/C3}$ columns. Thus, proteins eluting when C8-SO_3 (1%, w/v) is included in the mobile phase are considered to have intermediate affinity for IAM surfaces (Table 1). A 2% (w/v) C8-SO_3 concentration rapidly elutes pancreatic PLA_2. Increasing the alkyl sulfonate chain length from eight to ten carbons significantly increases the eluotropic strength of the mobile phase. Thus, after PLA_2 has been washed from the column with a 2% (w/v) C8-SO_3 mobile phase solution, a 1% (w/v) mobile phase solution of C10-SO_3 elutes the remaining proteins from the IAM surface. These proteins are considered to have very high affinity for IAM surfaces (Table 1).

Preliminary experiments demonstrated that C10-SO_3 had sufficient eluotropic strength to elute PLA_2 (Table 1). Thus, the first chromatographic experiment tested the utility of a C10-SO_3 step gradient to purify *Crotalus atrox* PLA_2. Fig. 2 shows the chromatogram after injecting *Crotalus atrox* PLA_2 into an ^{ether}IAM.PC$^{C10/C3}$ column and eluting with a step gradient of C10-SO_3 from 0 to 1.25% (w/v). Similarly to previous results [7–9,11,12], after loading any protein mixture on IAMs a "pass-through" peak containing non-retained proteins always elutes. Proteins in the "pass-through" peak are considered to have little or no affinity for IAM surfaces under the experimental loading conditions. The "pass-through" peak from loading the *Crotalus atrox* PLA_2 is labeled fraction 1 in Fig. 2; only one major protein eluted in the "pass-through" peak. Comparing the total proteins in the

Table 1
Classification of protein affinity for IAM surfaces based on elution with alkyl sulfonates

No affinity	Low affinity	Intermediate to high affinity
Proteins elute in the "pass-through" peak	Proteins elute with short-chain alkyl sulfonates	Proteins elute with long-chain alkyl sulfonates

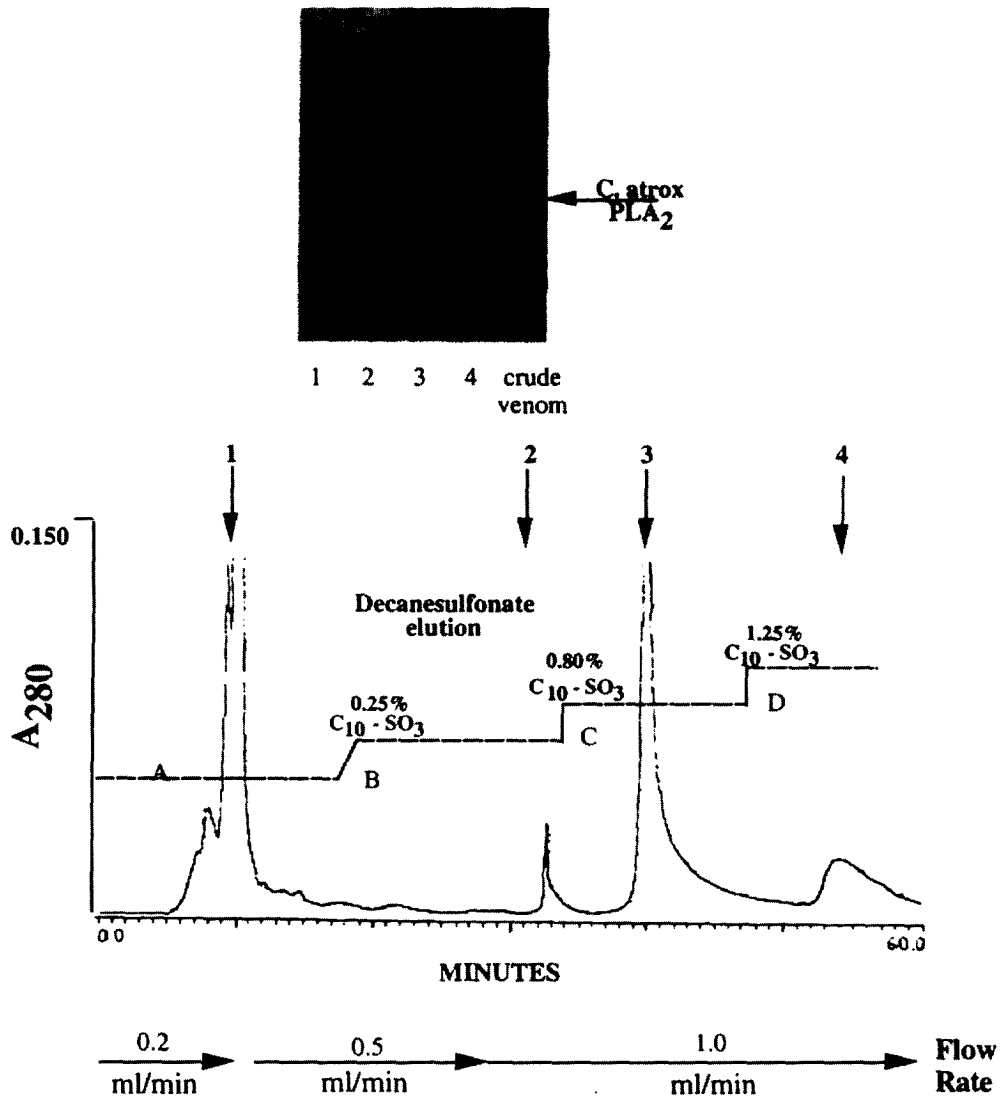

Fig. 2. Fractionation of *Crotalus atrox* crude venom on ^{ether}IAM.PC$^{C10/C3}$ surfaces using a step gradient from 0 to 1.25% (w/v) of the long-chain detergent C10-SO_3. A 750-μg amount of total protein in 200 μl of sample buffer was injected into the column. Mobile phase A contained 50 m*M* Tris (pH 7.2) and 25 m*M* KCl. Mobile phases B, C and D contained 0.25, 0.80 and 1.25% (w/v) of C10-SO_3 dissolved in mobile phase A, respectively. The detergent gradient (mobile phase A to B to C to D) is shown as a dashed line. The silver-stained 15% polyacrylamide gel shows that PLA_2 elutes with most of the injected proteins in fraction 3 which corresponds to mobile phase B [0.25% (w/v) C10-SO_3]. Few proteins eluted in fraction 4, which corresponds to C10-SO_3 concentrations above the CMC. Similar results were obtained for the $^{\delta G}$IAM.PC$^{C10/C3}$ stationary phase (not shown).

Crotalus atrox crude venom sample (Fig. 2, last PAGE lane) with the protein(s) eluting in the "pass-through" peak indicates that most of the proteins in the *Crotalus atrox* crude venom sample have a high affinity for the IAM surface. When the mobile phase eluotropic strength is increased by adding 0.25% (w/v) C10-SO_3 to the mobile phase, only three or four proteins elute (Fig. 2, lane 2). Hence this low concentration of C10-SO_3 can not remove most of the proteins adsorbed to the ^{ether}IAM.PC$^{C10/C3}$ surface, but selective fractionation of a few unknown proteins

in the sample occurred. Increasing the C10-SO_3 concentration to 0.8% in the mobile phase resulted in elution of virtually all proteins (Fig. 2, PAGE lane 3). All of the injected PLA_2 activity eluted at this detergent concentration but, as shown in Fig. 2 (PAGE lane 3), many contaminating proteins are present. Increasing the concentration of C10-SO_3 to 1.25% (w/v) resulted in only a few very high affinity proteins eluting from the IAM column (Fig. 2, lane 4). Based on the very high eluotropic strength of the 1.25% (w/v) C10-SO_3 mobile phase solution, we occasionally use this solution to wash and clean solution for IAM columns.

Since a step gradient of C10-SO_3 was unsuccessful at purifying PLA_2, a step gradient containing different alkyl chain lengths was tested (Fig. 3). Several preliminary experiments demon-

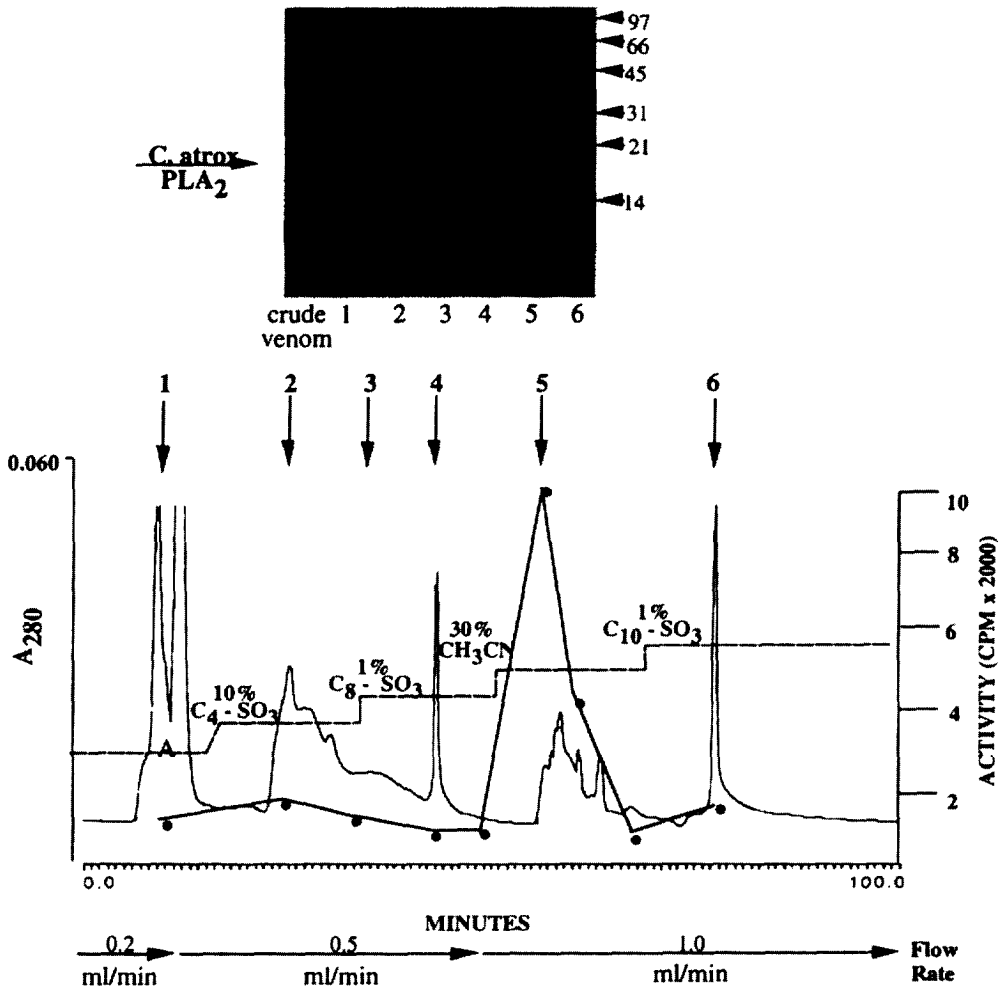

Fig. 3. Partial purification of *Crotalus atrox* PLA_2 on a 10 × 0.46 cm I.D. ^{ether}IAM.$PC^{C10/C3}$ column. A 746-μg amount of crude venom protein dissolved in 200 μl of sample buffer [25 m*M* $CaCl_2$–50 m*M* Tris (pH 7.6)] was injected into the column. Mobile phase A contained 50 m*M* Tris (pH 7.2) and 25 m*M* KCl. Mobile phase B contained 10% C4-SO_3 dissolved in mobile phase A. Mobile phase C contained 1% C9-SO_3 dissolved in mobile phase A. Mobile phase D contained 30% CH_3CN dissolved in mobile phase C. Mobile phase E contained 1% C10-SO_3 dissolved in mobile phase A. The detergent gradient is shown as a dashed line and PLA_2 activity as a solid line with closed circles. The inset shows a 15% polyacrylamide SDS gel stained with silver of the fractions eluting from the ^{ether}IAM.$PC^{C10/C3}$ column.

strated the changing the alkylsulfonate hydrocarbon group by only one or two carbons had little or no effect on the selective elution of proteins from the IAM surface. Consequently, the alkylsulfonate hydrophobicity was increased in increments of four carbons. The gradient consisted of C4-SO_3 (10%, w/v) then 1% (w/v) C8-SO_3, then 30% CH_3CN and finally 1% (w/v) C10-SO_3. The use of CH_3CN in the gradient was based on earlier experiments demonstrating that bovine pancreatic PLA_2 eluted with low concentrations of CH_3CN. Fig. 3 shows that 10% (w/v) C4-SO_3 eluted numerous proteins (Fig. 3, gel lane 2). Since PLA_2 activity did not elute in this fraction, this part of the gradient was highly successful at removing many contaminating proteins. Increasing the eluotropic strength to 1% (w/v) C8-SO_3 removed a few more contaminating proteins (gel lanes 3 and 4 in Fig. 3). When 30% CH_3CN was added to the 1% (w/v) C8-SO_3 mobile phase, all of the PLA activity eluted. Further, PLA_2 was the major protein as determined by gel electrophoresis analysis (Fig. 3, gel lane 5). After PLA_2 had eluted, a 1% (w/v) C10-SO_3 solution was used to elute several proteins (Fig. 3, gel lane 6). Proteins eluting with 1% (w/v) C10-SO_3 have a higher affinity for IAM surfaces than the target enzyme PLA_2. Thus, the elution profile shown in Fig. 3 was able to remove weakly adsorbed proteins, PLA_2 and high-affinity proteins from ^{ether}IAM.PC$^{C10/C3}$.

Purification of *Crotalus atrox* PLA_2 using the $^{\delta G}$IAM.PC$^{C10/C3}$ surface (Fig. 4) used the same elution conditions shown in Fig. 3 for the ^{ether}IAM.PC$^{C10/C3}$ surface. Comparing the chromatograms and protein patterns observed by PAGE analysis, Figs. 3 and 4 demonstrate that the two columns give very similar chromatographic results. For both IAM columns, (i) a "pass-through" peak containing only a few of the injected proteins was found (compare lane 1 in Figs. 3 and 4), (ii) the 10% (w/v) C4-SO_3 wash step removed most of the contaminating proteins from the columns (compare lane 2 in Figs. 3 and 4), (iii) the 1% (w/v) C8-SO_3 mobile phase eluted contaminating proteins but not PLA_2 (compare lane 4 in Figs. 3 and 4), (iv) PLA_2 eluted when 30% $CNCH_3$ was added to the 1% (w/v) C8-SO_3 mobile phase (compare lane 5 in Figs. 3 and 4) and (v) the 1% (w/v) C10-SO_3 mobile phase eluted many proteins that exhibit very high affinity for the IAM surface. Most important, the purity of PLA_2 in fraction 5 corresponding to a mobile phase of 30% $CNCH_3$–1% (w/v) C8-SO_3 is very similar for both columns. Based on scanning densitometry (Fig. 5), PLA_2 is a minor protein in the crude venom sample (4.5%), but, after IAM chromatography using either the $^{\delta G}$IAM.PC$^{C10/C3}$ surface or the ^{ether}IAM.PC$^{C10/C3}$ surface, PLA_2 is ca. 70% pure.

In this study, CH_3CN was used as the key mobile phase modifier to elute *Crotalus atrox* PLA_2 from IAM columns. Although snake venom PLA_2s are not denatured by CH_3CN, many other proteins are susceptible to denaturation with organic solvents. However, since we were primarily interested in PLA_2s, we did not extensively search for non-denaturing substitutes for CH_3CN. Nevertheless, during this work we found that 0.5% (w/v) octylphosphocholine lysolipids can be used as a substitute for CH_3CN (data not shown). Most important, the elution profile of the crude venom containing PLA_2 and other proteins was the same on both the $^{\delta G}$IAM.PC$^{C10/C3}$ and ^{ether}IAM.PC$^{C10/C3}$ surfaces when octylphosphocholine was substituted for CH_3CN.

As shown in Figs. 3 and 4 and Table 1, *Crotalus atrox* PLA_2 was partially purified ca. 255-fold from the IAM columns; only a few of high-molecular-mass proteins containing PLA_2 are present in fraction 5 shown in both Figs. 3 and 4. These contaminating high-molecular-mass proteins were removed using an anion-exchange column. Thus, fraction 5 obtained from the IAM column was injected into an anion-exchange column and pure PLA_2 eluted with ca. 0.2 *M* KCl (Fig. 6). The PLA_2 specific activity increased 77.2% after IAM chromatography and further to 751.2% after the anion-exchange chromatographic step. The data in Fig. 6 and Table 2 thus clearly show that a two-step purification protocol for *Crotalus atrox* PLA_2 is highly efficient.

All of the data in Figs. 1–6 were obtained on

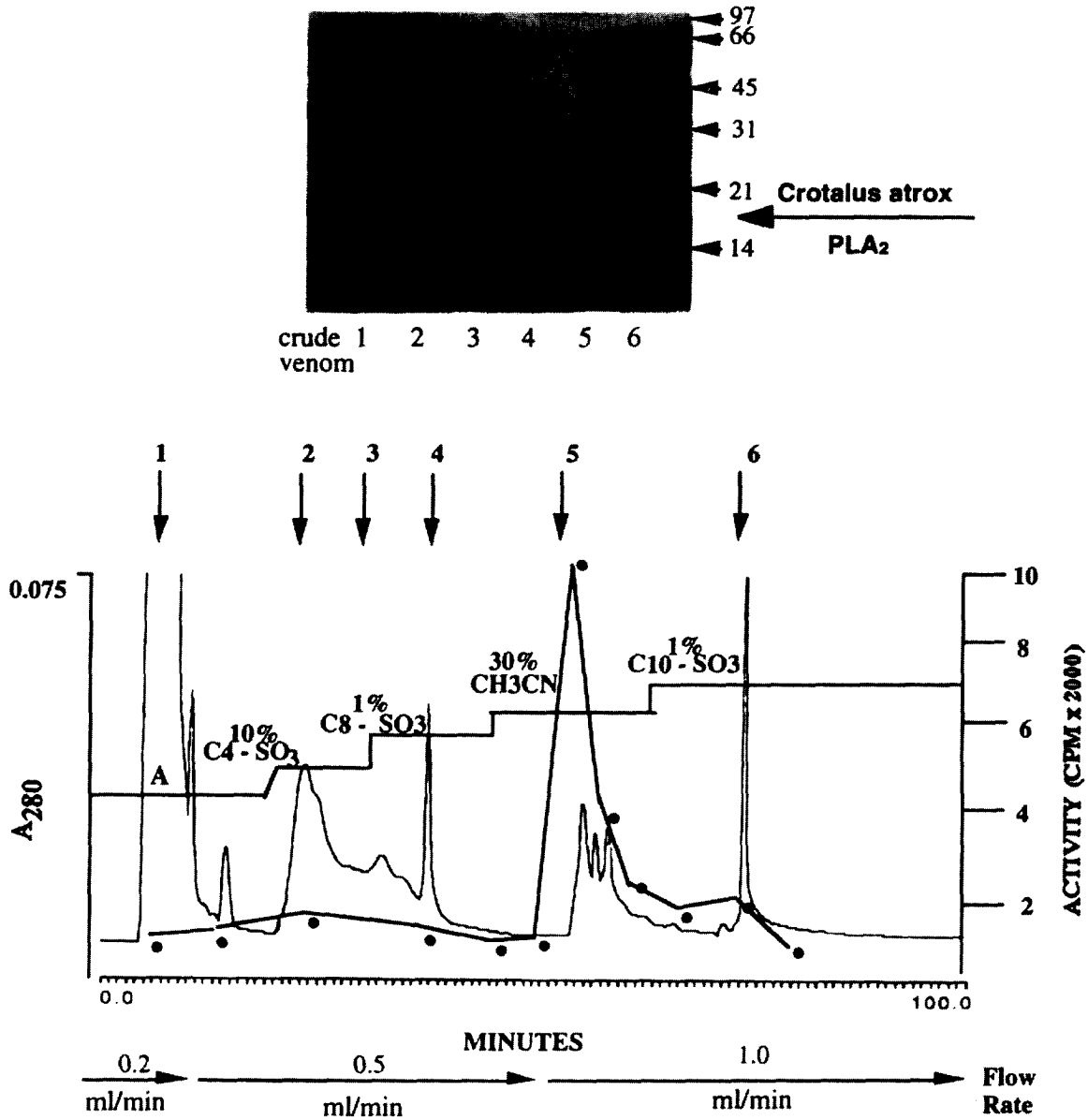

Fig. 4. Partial purification of *Crotalus atrox* PLA_2 using a 10×0.46 cm I.D. $^{\delta G}IAM.PC^{C10/C3}$ column. Gradient and SDS-PAGE conditions were identical with those used to obtain the chromatogram in Fig. 3.

analytical-size IAM columns. However, scale-up is essential for obtaining sufficient enzymes to study the functional activity of enzymes. We therefore attempted to scale up the PLA_2 snake venom purification on the $^{\delta G}IAM.PC^{C10/C3}$ column using the same elution conditions shown in Figs. 3 and 4. The important observation is that lane b in Fig. 7 shows that PLA_2 was purified fairly well on scale-up and PLA_2 eluted at the same mobile phase elution conditions, i.e., 30% CH_3CN–1% (w/v) C8-SO_3.

Bovine pancreatic PLA_2 was purified to homogeneity from crude pancreatic tissue using an $^{ether}IAM.PC^{C10/C3}$ column and a 7% (v/v)

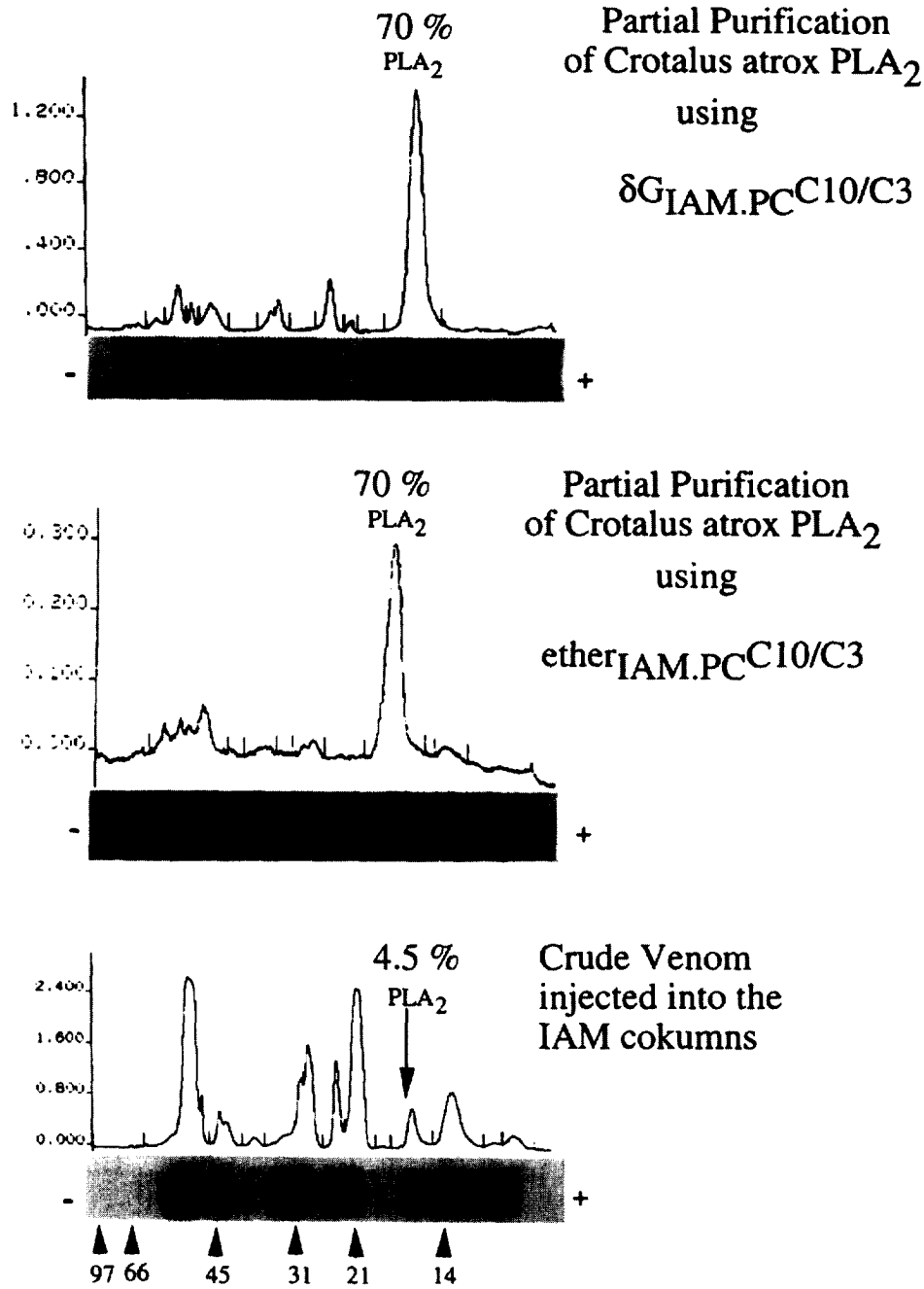

Fig. 5. Densitometric scans of the SDS-PAGE gels shown in Figs. 3 and 4 of fraction 5. Fraction 5 contained PLA_2 partially purified using IAM chromatography. For comparison, the bottom densitometric scan corresponds to the crude venom material injected into the IAM columns. IAM chromatography (upper and centre scans) resulted in ca. 70% pure PLA_2. The crude venom of *C. atrox* contains about 4.5% PLA_2 (bottom scan).

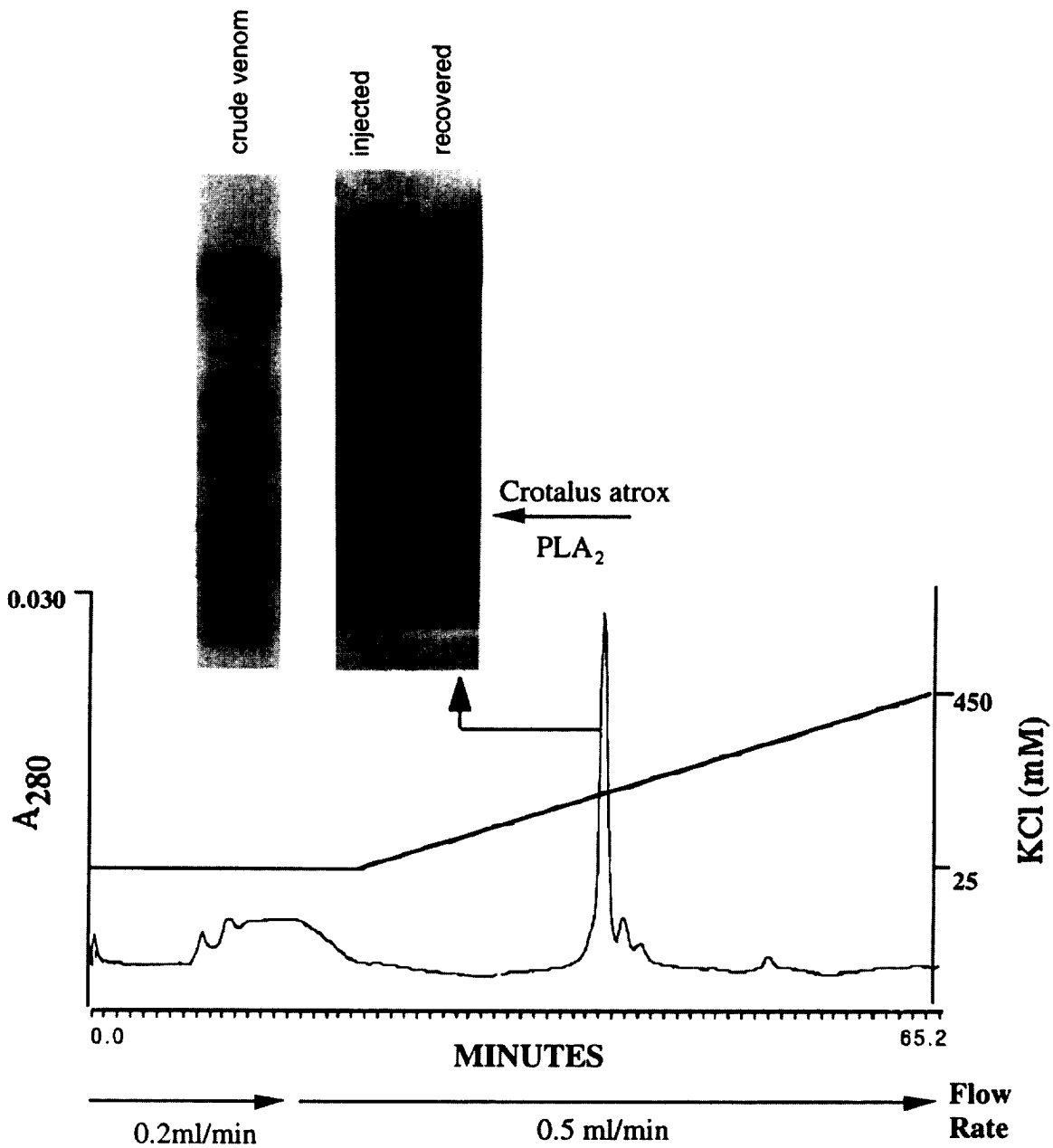

Fig. 6. Purification of *Crotalus atrox* PLA_2 to SDS-PAGE homogeneity using a strong anion exchanger. Acetonitrile fractions (i.e., fractions 5 in Figs. 3 and 4) from IAM columns contained ca. 70% pure PLA_2. After removing acetonitrile by evaporation, 52.1 μg of total protein obtained from the IAM column were injected into the anion-exchange column. Pure PLA_2 eluted at about 0.2 *M* KCl using a KCl gradient. The inset shows a 15% silver-stained polyacrylamide gel comparing the injected proteins with the recovered proteins. The diffuse bands above PLA_2 are an artifact that was present in all gel lanes. PLA_2 is the only protein in the gel.

Table 2
Purification of *Crotalus atrox* PLA_2 using $^{\delta G}IAM.PC^{C10/C3}$ and $^{ether}IAM.PC^{C10/C3}$ columns

Column	PLA_2 specific activity in crude venom (μmol/mg min)	PLA_2 specific activity after IAM chromatography[a] (μmol/mg min)	Increase in specific activity (%)	Total protein injected into IAM column (mg)	Total protein in CH_3CN fraction[a] (μg)	PLA_2 in CH_3CN fraction[b] (μg)	PLA_2 recovered from IAM column (%)
$^{\delta G}IAM.PC^{C10/C3}$ (analytical-scale purification)	1.37	352	256.9	0.746	41.2	27.6	82
$^{ether}IAM.PC^{C10/C3}$ (analytical-scale purification)	1.28	326	254.6	0.746	41.8	30.1	88
$^{\delta G}IAM.PC^{C10/C3}$ (preparative-scale purification)				8.36	702.0	338.4	89

[a] PLA_2 activity was measured in pooled chromatographic fractions that were obtained when CH_3CN was included in the mobile phase. See Figs. 3 and 4 for the mobile phase gradient and the fractions containing PLA_2. The percentage of PLA_2 recovered from the IAM column is based on the amount of protein in this pooled fraction.
[b] PLA_2 content was measured by scanning densitometry as described under Experimental. See Fig. 5.

CH_3CN gradient [12]. This should be contrasted with the purification of *Crotalus atrox* PLA_2, which required 30% acetonitrile. This indicates that the amount of acetonitrile required to elute a particular PLA_2 from a specific IAM column must be determined experimentally. Futhermore, based on IAM chromatography in the presence and absence of Ca^{2+}, the binding of PLA_2s to IAMs is a protein surface–IAM surface interaction. In other words, the IAM binding site does not utilize the active site of PLA_2. Evidence for this concept is obtained from affinity purification of PLA_2 using affinity surfaces that utilize the PLA_2 phospholipid binding site. For instance, monomers of pancreatic PLA_2 productively hydrolyze phospholipids with a Ca^{2+}-dependent catalysis which indicates that Ca^{2+} participates in the affinity of the enzyme for the phospholipid in the enzyme active site [20]. Affinity purification of PLA_2s utilized this observation by performing the protein loading step in the presence of Ca^{2+} and the affinity elution step using EDTA to

Table 3
Purification of *Crotalus atrox* PLA_2 using $^{\delta G}IAM.PC^{C10/C3}$ and a strong ion exchanger

Column	PLA_2 specific activity injected into the column (μmol/mg min)	PLA_2 specific activity in pooled fractions eluting from the column[b] (μmol/mg min)	Increase in PLA_2 specific activity[c] (%)	Total protein injected into the column (μg)	Total PLA_2 protein after column chromatography (μg)
$^{\delta G}IAM.PC^{C10/C3}$ (first column; partial purification)	2.98[a]	230.0	77.2	2010.0	156.1
Anion exchange	230.0	2238.0	751.2	52.1	8.0

Partial purification of PLA_2 was achieved by the IAM column and complete purification was obtained using a strong anion-exchange column.
[a] This is also the PLA_2 specific activity found in the crude venom.
[b] See Fig. 4 for the IAM chromatographic conditions and Fig. 6 for the anion-exchange chromatographic conditions.
[c] The percentage increase in PLA_2 specific activity was calculated relative to the PLA_2 specific activity in the crude venom which is given in column 2 as 2.98 μmol/mg min.

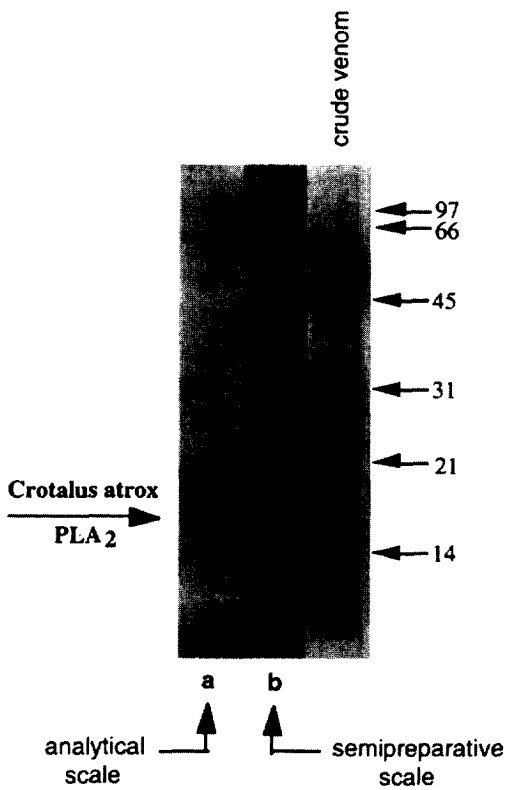

Fig. 7. Comparison of PLA_2 purity obtained after analytical IAM chromatography (0.8 mg of protein loaded per gram of $^{\delta G}IAM.PC^{C10/C3}$) and (b) semi-preparative IAM chromatography (3.2 mg of protein loaded per gram of $^{\delta G}IAM.PC^{C10/C3}$). Analytical columns were 10 cm × 0.46 cm I.D. whereas semi-preparative columns were 4 × 1 mm I.D. Mobile phase conditions for the semi-preparative chromatography were identical with those in Fig. 3 for the analytical column.

remove both Ca^{2+} and PLA_2 from the column [21–23]. These $\pm Ca^{2+}$ elution conditions have no effect on the affinity of pancreatic PLA_2 for $^{ether}IAM.PC^{C10/C3}$ surfaces (not shown). This indicates that the binding mechanisms between IAM and PLA_2s do not involve the enzyme active site, but rather the protein surface and the IAM surface.

In addition to pancreatic (group I) and Crotalus (*Crotalus atrox* PLA_2; group II) PLA_2s, we evaluated the IAM affinity interactions of *B. multicinctus* and bee venom (*A. mellifera*) PLA_2s. All PLA_2 samples tested showed that PLA2 strongly adsorbed to IAM surfaces and required eluotropic strengths ≥2% C8-SO_3 to elute the particular PLA_2 injected into the IAM column (data not shown). The detergent conditions that wash weakly adsorbed proteins and elute these different PLA_2 enzymes from IAMs seem to be similar for all PLA_2s tested. This suggests that the elution conditions described in this paper may be used for the purification of PLA_2s from different biological samples.

4. Conclusions

The main objective of this work was to increase our understanding of the binding of PLA_2 enzymes to IAM surfaces. We demonstrated that the affinity recognition of PLA_2 enzymes for $^{ether}IAM.PC^{C10/C3}$ and $^{\delta G}IAM.PC^{C10/C3}$ surfaces is virtually identical. This indicates that phospholipid ligands forming the immobilized artificial membrane surface do not require the glycerol backbone to exhibit a high affinity for PLA_2 enzymes. The biological importance of phospholipases and the concept that the interfacial membrane recognition of phospholipases suggest that IAMs may function as a general affinity membrane surfaces for purifying not only PLA_2 enzymes but also other interfacial binding enzymes such as phospholipase A_1, phospholipase C and phospholipase D.

Acknowledgements

This work was supported in part by the NIH (AI33031 and 2R446M3022-02) and NSF (CTS 9214794).

Abbreviations

BSA	bovine serum albumin
C4-SO_3	butylsulfonate (sodium)
CMC	critical micelle concentration
DOC	deoxycholate
DPPC	dipalmitoylphosphatidylcholine

C10-SO_3	decylsulfonate (sodium)
IAM	immobilized artificial membrane
^{ether}IAM.PC$^{C10/C3}$	An IAM surface prepared from a monoalkylated phosphatidylcholine ligand and end-capped with decanoic anhydride (C10 groups) and then propionic anhydride (C3 groups)
$^{\delta G}$IAM.PC$^{C10/C3}$	An IAM surface prepared from a phosphatidylcholine (PC) ligand that does not contain a glycerol backbone. The PC polar headgroup is linked directly to an alkyl chain
C8-SO_3	octylsulfonate (sodium)
PAGE	polyacrylamide gel electrophoresis
PL	phospholipid(s)
PC	phosphatidylcholine
PLA_2	phospholipase A_2
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid

References

[1] S. Ong, S.J. Cai, C. Bernal, D. Rhee, X. Qiu and C. Pidgeon, Anal. Chem., 66 (1994) 782–792.

[2] X. Qiu, S. Ong, C. Bernal, D. Rhee and C. Pidgeon, J. Org. Chem., 59 (1994) 537–543.

[3] X. Qiu and C. Pidgeon, J. Phys. Chem., 97 (1993) 12399–12407.

[4] R.J. Markovich and J.M. Stevens, Anal. Biochem., 182 (1989) 237–244.

[5] D.R.M. Rhee, W.G. Chae, X. Qiu and C. Pidgeon, Anal. Chim. Acta, 297 (1994) 377–386.

[6] X.M. Zhang and I.W. Wainer, Tetrahedron Lett., 34 (1993) 4731–4734.

[7] H. Thurnhofer, J. Schnabel, M. Betz, G. Lipka, C. Pidgeon and H. Hauser, Biochim. Biophys. Acta, 1064 (1991) 275–286.

[8] C. Pidgeon, J. Stevens, S. Otto, C. Jefcoate and C. Marcus, Anal. Biochem., 194 (1991) 163–173.

[9] S.J. Cai, R.S. McAndrew, B.P. Leonard, K.D. Chapman and C. Pidgeon, J. Chromatogr. A, 696 (1995) 49–62.

[10] F.M. Alvarez, C.B. Bottom, P. Chickale and C. Pidgeon, in T. Ngo (Editor), Molecular Interactions in Bioseparations, Plenum Press, New York, 1993, pp. 151–167.

[11] S. Otto, C. Marcus, C. Pidgeon and C. Jefcoate, Endocrinology, 129 (1991) 970–982.

[12] C. Pidgeon, S. Cai and C. Bernal, J. Chromatogr. A, 721 (1996) 213.

[13] A. Achari, D. Scott, P. Barlow, J.C. Vidal, Z. Otwinowsky, S. Brunie and P.B. Sigler, Cold Spring Harbor Symp. Quant. Biol., 12 (1987) 441–452.

[14] C. Pidgeon and U.V. Venkatarum, Anal. Biochem., 176 (1989) 36–47.

[15] E.A. Dennis, Phospholipases, Vol. 197, Academic Press, San Diego, 1991.

[16] R.M. Kramer, C. Hession, B. Johansen, H. Gretchen and P. McGray, J. Biol. Chem., 264 (1989) 5768–5775.

[17] M.L.W. Hearn and B. Grego, J. Chromatogr., 296 (1984) 309–319.

[18] R.A. Barford and B.J. Sliwinsky, Anal. Chem., 56 (1984) 1554–1556.

[19] P.A.M. Mukerjee and K.J. Mysels, Critical Micelle Concentrations of Aqueous Surfactant Systems, Secretary of Commerce, Washington, DC, 1971.

[20] B.W. Dijkstra, J. Drenth and K.H. Kalk, Nature, 289 (1981) 604–608.

[21] A. Aarsman, F. Neys and H. Van den Bosch, Biochim. Biophys. Acta, 792 (1984) 363–366.

[22] S. Tahir and R.C. Hilder, Anal. Biochem., 135 (1983) 332–334.

[23] K. Saito, J. Sugatani and T. Okamura, Methods Enzymol., 197 (1991) 446–456.