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Multiple Forms of Phospholipase A_2 in Arthritic Synovial Fluid

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Phospholipase A_2 (PLA_2) has been purified to homogeneity from human arthritic synovial fluid. The activity resolved into multiple peaks by preparative HPLC. The most abundant peak (A) was present in synovial fluid from patients with rheumatoid arthritis, osteoarthritis, and psoriatic arthritis. A second major peak (B) was variable and lower in relative abundance, but was distinguishable from peak A by its stimulated activity in the presence of either 0.5 M Tris or 0.1% sodium deoxycholate (DOC), in addition to its longer HPLC column retention time. Both peaks required Ca^{2+} and showed optimal activity in DOC/phosphatidylcholine (PC) mixed micelle assays between pH 8.0 and 9.0. Both peaks showed higher activity with PC as substrate than with PI, however peak A exhibited higher activity with PE than PC. Upon preparative SDS-polyacrylamide gel electrophoresis, both peaks of PLA_2 activity were resolved as proteins of approximately 14,000 Da. The N-terminal sequence obtained from purified peak A material matched that of a recent similar isolate (Hara *et al.* (1988) *J. Biochem.* 104, 326-328).

The central role played by a variety of eicosanoid products in inducing and maintaining chronic inflammatory disease states has become widely appreciated. In the past several years some of the enzymes participating in eicosanoid production and regulation have been identified, characterized, and cloned. These enzymes include cyclooxygenase (*1*), lipoxygenase (*2*), and phospholipase C (*3*). While primary sequence information has been obtained for the phospholipase A_2 found in human pancreas (*4, 5*), less is currently known about the phospholipase A_2 enzymes which release arachidonate from membrane phospholipids which, in turn, initiate the metabolic cascade. Several mammalian cellular (non-pancreatic) PLA_2 enzymes have been identified and characterized (*6-12*), however it is presently unclear which PLA_2 enzymes are relevant in human inflammatory disease. It is even possible that multiple forms of PLA_2 play distinct roles in different types of inflammatory diseases.

Recent progress on the purification and characterization of a PLA_2 enzyme which accumulates to high levels in the synovial fluid of rheumatoid arthritis patients (*13-19*) suggests this enzyme may participate in this chronic inflammatory disease. Over the past few years, we (*20, 21*) and several other groups (*12, 18, 19*) have purified and characterized this enzyme. Here, using improved fractionation procedures, we present evidence that human synovial fluid contains at least two enzymatically distinct PLA_2 activities. The N-terminal amino acid sequence of the more abundant of the two activities (peak A) is identical to a recently reported similar isolate from rheumatoid synovial fluid (*12*). The second activity (peak B) exhibits distinct biochemical properties and may represent a modified form of the peak A enzyme or may be an entirely different enzyme present in these fluids. The presence of multiple and biochemically distinct PLA_2s may have consequences in the pathology, diagnosis, and therapeutic treatment of these disorders.

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METHODS

Standard assay conditions consisted of 50 mM Tris, pH 8.0, 150 mM NaCl, 5.0 mM $CaCl_2$, 0.04% (1.0 mM) sodium deoxycholate (DOC), and 0.22 nmol (0.015 μCi) of 1-stearoyl-2-[1-^{14}C]arachidonyl-L-3-phosphatidylcholine (PC, Amersham #CFA.655) as substrate, incubated at 37°C for 30 min. The substrate was prepared by dissolving freshly-dessicated PC in 2% DOC, which was then diluted 50-fold to the appropriate concentration in assay buffer. The 50 μl reaction was started by the addition of prewarmed substrate and terminated by the addition of 10 μl 8 M acetic acid. Fifty microliters of the reaction mixture were spotted and dried onto Whatman thin-layer chromatography plates (Silica gel 60A #LK6D), and the plates were chromatographed using chloroform-methanol-acetic acid (90 : 10 : 1) as a solvent. The dried plates were autoradiographed overnight, or alternatively the bands corresponding to product (arachidonate) and substrate (PC) were scraped and counted in scintillation fluid. The other substrates used, PI (1-stearoyl-2-[1-^{14}C]arachidonyl-L-3-phosphatidylinositol; NEC-787) and PE (1-palmitoyl-2-[1-^{14}C]arachidonyl-L-3-phosphatidylethanolamine; NEC-783), were obtained from DuPont/NEN. Under these conditions, the assay was linear up to 30% hydrolysis.

The initial purification of the enzyme was performed essentially as previously described (*20, 21*). Pooled synovial fluid from several patients with rheumatoid arthritis was dialyzed overnight in 5 mM acetate buffer, pH 5.0. The precipitate was collected and redissolved in 0.5 M acetate buffer and loaded onto either an octyl- or CM-Sephadex

column and eluted with 0.2 M Tris, pH 8.5. The active fractions were reconstituted in 2.0 M NaCl, 50 mM Tris, pH 8.5, and concentrated by dialysis. The post-CM Sephadex material was loaded onto a 4.0×0.5 cm reverse-phase C-4 HPLC column, and eluted with a 15-60% acetonitrile gradient in the presence of 0.1% trifluoroacetic acid. Aliquots of the elution fractions were assayed, and the active fractions were pooled and lyophilized overnight in a siliconized Falcon #2059 tube. The two peaks of activity described in the text were purified separately from this point on.

Material to be further purified was resuspended in 1X PAGE loading buffer (2.3% SDS, 50 mM Tris, 10% glycerol), heated at 90°C for 3 min, and loaded onto a 15% acrylamide minigel. ^{125}I-labeled (40,000 dpm) porcine propancreatic PLA_2 was included within the sample as an autoradiographic marker. After electrophoresis, the gel was autoradiographed for 30 min, and then cut into 1.0 mm slices, using the autoradiogram as a cutting guide. The slices were crushed and the activity was eluted in 10 mM *n*-ethyl-morpholine acetate for 1-2 d. Assays were performed on 1.0 μl of the eluate after 60 min of elution at 25°C, and an activity profile was obtained. Active fractions were spotted directly onto quaternary amine glass fiber filter paper. The filters were washed four times in the same buffer, 5 min each, and dried. Sequence analysis was performed *via* Edman degradation on an Applied Biosystems gas phase sequencer.

Radioiodination of porcine propancreatic PLA_2 (Sigma #P-9139) was performed using the chloramine T method (*22*), and unincorporated ^{125}I label was removed by differential elution from C18-RP Sep-Pak column (Waters, Inc.).

RESULTS

As shown in Fig. 1, post-CM Sephadex material was analyzed by reverse-phase HPLC and assayed under standard conditions. Two major peaks of activity were routinely observed, eluting at 24% (peak A) and 28% (peak B) acetonitrile. An additional minor peak of activity (peak C), eluting near 40% acetonitrile, was seen in some but not all extracts. The total activity represented by the eluted peaks was typically near 100% of the activity loaded. Under standard assay conditions, the peak B activity varied from 1 to 33% of the total activity but was similar in repetitive analyses using the same starting material.

To determine whether the difference in chromatographic behavior between the peaks was a stable phenomenon, we rechromatographed the peaks. The separately-pooled peak material was lyophilized and resuspended in 50 mM Tris, pH 8.0, containing 0.2% DOC. The detergent was necessary to completely solubilize the activity from the walls of the tube. The material was then reapplied to the column and eluted using identical gradients. As seen in Fig. 1, the peaks retained their individual chromatographic behavior and no interconversion of the two peaks was noted.

It has previously been reported that the PLA_2 activity present in rheumatoid synovial fluid shows variable activity when measured using different assay conditions (*18*). In addition, both ionic strength and changes in detergent concentration have been used previously to compare differences in enzyme kinetics of other purified PLA_2s (*23, 24*).

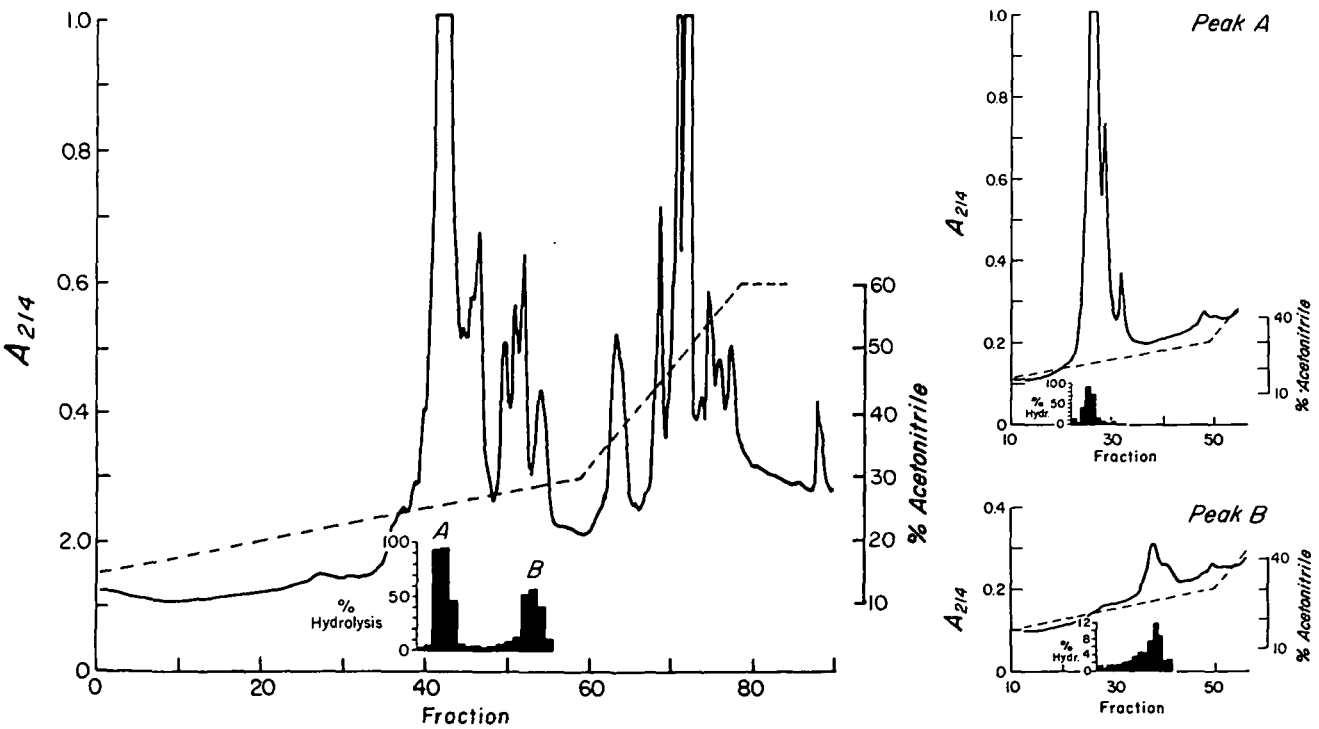

Fig. 1. **HPLC of post-CM synovial fluid PLA_2.** One hundred microliters (50 μg protein) of post CM-Sephadex material was loaded onto a RP-C4 cartridge column and eluted with a 15-60% acetonitrile gradient in 0.1% trifluoroacetic acid. Fifty-microliter aliquots of the 0.5 ml fractions were lyophilized and assayed for PLA_2 activity under standard conditions described in "METHODS." The two peaks of activity, A (left) and B (right) are represented in the histogram inset below the tracing. At right, the pooled peak material from peak A (top) and B (bottom) was rechromatographed separately using identical gradients.

In order to determine if both peaks displayed the same enzymatic characteristics or if there may be additional forms of the enzyme not apparent under our standard assay parameters, we assayed all of the gradient fractions at elevated Tris and deoxycholate concentrations as well as at various pHs.

The effect of a 10-fold increase in Tris concentration (0.5 M) in an otherwise standard assay system is shown across a typical gradient in Fig. 2. The activities of the fractions containing peak A activity were inhibited while the peak B fractions were stimulated. When the separated peaks were individually examined in titration experiments where the enzyme concentrations were maintained within the linear range of the assay, the 0.5 M/0.05M Tris-stimulation factors were 0.17 for peak A and 8.0 for peak B. A similar but less dramatic difference between the peaks was observed by raising the DOC concentration 2.5-fold to 0.1%, seen also in Fig. 2. In this case the peak A enzyme was only moderately affected while fractions across the B enzyme peak were again stimulated. The greatest effect was seen in fractions whose activities, under standard conditions, are within the linear range of the assay (under 30% hydrolysis). As reported by others (*18*), the enzyme activity also depended upon the NaCl concentration. Varying NaCl concentrations had differential effects on the peaks, and these effects depended upon the concentrations of Ca^{2+} and Tris (data not shown). Under the conditions examined, no additional peaks of activity other than the A and B enzymes were observed.

In order to confirm whether both peaks of activity represented Ca^{2+} dependent alkaline activity as described previously for synovial fluid PLA_2 (*21*), the gradients also were assayed at pHs ranging from 6.0 to 9.0, while holding other assay parameters constant (Fig. 3). Both peaks were optimally active at pH 8.0 to 9.0, with lesser but substantial activity present at lower pH. Although this is not as apparent for the peak fractions corresponding to the "A" enzyme (fractions 46 and 47) due to assay non-linearity, it is clear that fractions on both sides of the peak (*i.e.* fractions 45 and 48) which are in the linear range, show a clear preference for pH 8-9. No additional peaks of activity were detected at pH 6.0 or 7.0, nor was any activity detected across the gradients in the presence of 25 mM EDTA. The effects of substrate head group substitution were tested by measuring the activities of each peak separately with PC, PE, and PI under standard conditions and equivalent substrate molarity. When the data were normalized to the activity observed with PC as substrate, the relative substrate activities were PE 4.0>PC 1.0>PI 0.9 for peak A and PC 1.0>PE 0.34>PI 0.26 for peak B (data not shown).

Final purity was achieved by relying upon the high resolution of SDS-polyacrylamide gel electrophoresis. The pooled peaks were purified separately on non-reducing gels. Radioiodinated porcine pancreatic PLA_2 zymogen was used as a convenient gel marker because of its size and blocked N-terminus which precluded contamination of the sequencing data. As shown in Fig. 4, a portion of the preparative gel was silver stained, and the PLA_2 activity and ^{125}I label which eluted from each gel slice was assayed. This method permitted quantitative recovery of activity, which was associated with an approximately 14 kDa band for both peaks. The eluted material displayed similar inhibition (peak A) and stimulation (peak B) in the presence of 0.5 M Tris as the post-HPLC peak material. Calculation of the fold purification from the starting material was made ambiguous by the presence of the multiple forms until after the HPLC step. In addition, since the two major forms have different optimal assay conditions, it was impossible to accurately quantitate the activities of the two peaks simultaneously in the same mixture. Allowing for the peak B activity present in the earlier purification steps (see purification table in Ref. *20*), we estimate that the HPLC step added a threefold increase in purification and specific activity, resulting in a final purification of about 12,000-fold. Post-gel purified peak A enzyme when assayed with PC as substrate at enzyme concentrations in the linear range of the assay exhibited a specific activity of 22 μmol/

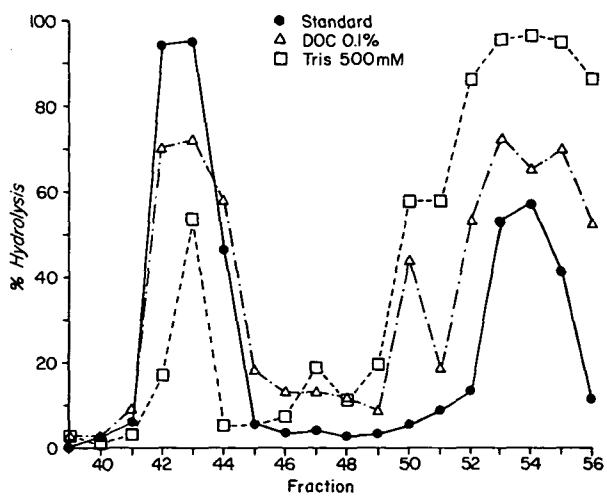

Fig. 2. **Effects of DOC and Tris.** Similar to Fig. 1, except PLA_2 activity was assayed under standard conditions (circle), and in the presence of standard conditions plus 0.1% DOC (triangle) or 0.5 M Tris (square).

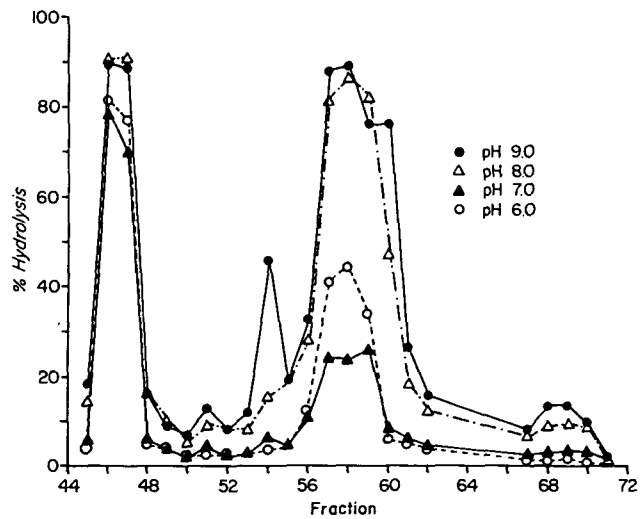

Fig. 3. **Effects of pH.** Similar to Fig. 1, except activity was measured in the presence of 50 mM Tris-HCl at pH 6.0 (open circle). 7.0 (solid triangle), 8.0 (open triangle), and 9.0 (solid circle). A small third peak of activity, seen here as fractions 67-70 (peak C), was detected in some extracts.

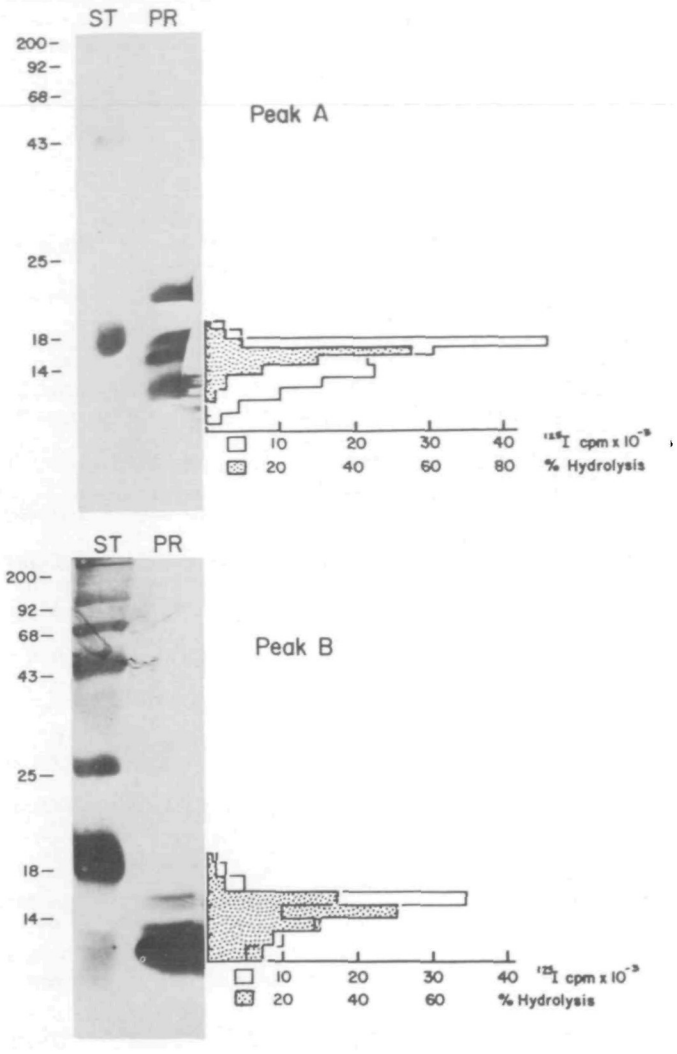

Fig. 4. **Preparative PAGE of PLA_2 peaks.** Pooled post-HPLC peak A (top) and B (bottom) was electrophoresed on a preparative non-reducing gel, a portion of the gel was removed for silver staining (at left), and the remainder of the gel was sliced horizontally. The left lane of both gels contained molecular weight standards (ST), comprised of cytochrome *c* (12.3 kDa), β-lactoglobulin (18.4 kDa), α-chymotrypsinogen (27.7 kDa), ovalbumin (43.0 kDa), bovine serum albumin (68.0 kDa), phosphorylase B (95.5 kDa), and myosin (200 kDa). The right lane was a portion of the preparative lane (PR). Eluted PLA_2 activity (stippled) and ^{125}I cpm (open) from the gel slices is plotted on the right.

mg/30 min. This estimate was made using standard assay conditions and the protein yield was based upon average N-terminal PTH-amino acid signal amplitude calculated from amino acid sequencing of the sample.

After transfer of the gel purified peak A fractions to quaternary amine-modified glass fiber paper and washing with volatile buffer (see "METHODS"), the material remaining bound to the filter was sequenced by N-terminal Edman degradation. The 30-residue amino acid sequence obtained for peak A is shown in Fig. 5, and was aligned with other mammalian PLA_2s. Since no signals were detected at residues 27 and 29 with non-alkylated material, we assumed these residues were most likely Cys residues, based upon homology to the other PLA_2 enzymes shown, and have indicated them as such. The sequence obtained for the peak

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                    1        10        20        30        40
h RASF-A            ?LVNFHRMIK-LTTGKEAALSYGFYGC?CG?GG?G
h "non Panc" (11)   SFWQFQRRVK-HITGRSAFFSYGYGCYCGLGDKGIPVDDTDR
h Panc (4,5)        AVWQFRKMIKCVIPGSDPFLEYNNYGCYCGLGGSGTPVDELDK

p ileum (6)         DLLNFRKMIK-LKTGKAPVPNYAFYGCYCGLGGKGSPKDATD?
rab ascites (7)     HLLDFRKMIR-YTTGKEAT?SYGAYGCSCGVGGR?APK?A
rat (8)             ?LLEPGQMIL-FKTGKRADVSYGFYGCHCGVGGRGSPKDATDE

C. atrox (25)       SLVQFETLIM-KIAGRSGLLWYSAYGCYCGWGGHGLPQDATDR
A. pisc (26)        SVLELGKMIL-QETGKNAITSYGSYGCNCGVGHRGQPKDATDR
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Fig. 5. **N-terminal PLA_2 sequence homology.** The sequence of peak A material (h RASF-A) was aligned with other relevant PLA_2 sequences, including porcine ileum (p ileum; Ref. *6*), rabbit ascites (rab ascites; Ref. *7*), rat platelet (*8*), spleen (*9*), and peritoneal exudate (rat, Ref. *10*), "non-pancreatic" (NP; Ref. *11*), human pancreas (h panc; Refs. *4* and *5*), and *Crotalus atrox* (C. atrox; Ref. *25*) and *Agkistrodon piscivorus* "K-49" venom (A. pisc; Ref. *26*). Conserved residues are underlined.

A enzyme has been verified by cloning of the cDNA (*33, 34*).

DISCUSSION

The resolution of the PLA_2 activity from human synovial fluid into separate activities was a fortuitous result of the choice of reverse-phase HPLC as a purification step. The difference in HPLC column retention time was stable under working conditions as demonstrated by rechromatography (Fig. 1). In addition, the enzymological differences between the two activities with regard to optimal Tris, DOC, and NaCl concentrations, and substrate head group were quite substantial. One possibility for these differences is that one of the forms is stably bound to another protein through the HPLC step which modifies the enzymatic behavior in the observed manner. For this explanation to be true the modifying protein would have to be the same size as the PLA_2 (or another PLA_2 monomer) since both forms eluted from the preparative gel at similar molecular weights yet retained their differential response to Tris concentration. A chemical modification such as acylation (*27*) involving one of the forms could also explain these differences.

Another explanation is that the two forms represent discrete enzymes. At present, however, adequate amounts of the peak B material for sequence determination will require both a more abundant source of peak B and an additional purification step. We therefore can not yet distinguish between these three possibilities.

The differential responses of the two peaks to Tris, DOC, NaCl, and substrate head group could represent direct effects upon (or preferences of) the enzymes themselves, or more likely may reflect effects upon the substrate. Such substrate effects could be related to a change in micellar structure or perhaps to altered distribution and conformation of the substrate within the micelle. Since the assay conditions are at or very near the critical micelle concentration of DOC (*28*), this would seem a likely possibility. If this is the case, the two peaks then appear to exhibit different substrate conformational preferences, reminiscent perhaps of the preference for micellar substrates exhibited by human pancreatic PLA_2 *versus* its zymogen (*29*).

These differences in activity and/or substrate specificity between the two peaks raises the possibility that each may have different functions *in vivo* in the pathogenesis of

disease. In the *in vivo* situation, differences in the conformation of available substrate may regulate the liberation of free arachidonic acid for eicosanoid production. The present results suggest that variations in the relative levels of the two enzyme forms could modify this response and itself represent a separate regulatory mechanism. In this regard, quantitation of the relative amounts of the enzyme forms present in certain inflammatory diseases could prove of clinical or diagnostic value. Of interest is that our initial results suggest fluid from osteoarthritis and psoriatic arthritis patients contain relatively more peak B material than fluid from rheumatoid arthritis (*30*). Fortunately, the differential responses to changes in Tris or DOC concentration or substrate head group preference allows identification of the two activities within complex mixtures, once separated chromatographically. On the other hand, the presence and distinct behavior of these peaks adds a new complexity to previous observations (*13-19*) employing direct measurement of the total PLA_2 enzyme levels present in serum or synovial fluid.

The N-terminal sequence obtained for peak A shows marked conservation of structure when compared to other PLA_2s, and particularly the mammalian "cellular" type II enzymes (*6-12, 31, 32*). Several structural features worthy of note are the lack of Cys_{11}, consistent with other Type II enzymes, the conservation of active site residues Phe_5, Met_8, Ile_9 and the Ca^{2+}-binding loop sequence Tyr_{28} $Gly_{29}Cys_{30}Tyr_{31}$- - -Gly_{33}. Its overall homology to other mammalian non-pancreatic enzymes is high in the Ca^{2+}-binding loop area including residues 25-35, yet quite divergent near the amino terminus (residues 2-24). If the other cellular Type II enzymes identified to date (Refs. *6-10*) represent a PLA_2 analogous to the peak A enzyme in their respective species, then the degree of cross-species divergence is relatively higher than was observed with the other PLA_2 forms. For example, over the first 42 amino acids the cellular type II PLA_2 from rat (*8-10*), porcine (*6*), and rabbit (*7*) share 73, 66, and 70% homology respectively with the human synovial peak A enzyme. In contrast, the rat and porcine pancreatic PLA_2s share 88 and 81% homology with the human pancreatic PLA_2 in this same region of the molecule. One possibility is that PLA_2 enzymes with primarily inflammatory functions may have developed a greater level of species specificity than those (such as the "pancreatic" form) which may be of primarily digestive function. A full understanding of this issue must await the entire sequences of the other forms as well as knowledge of their respective sites of synthesis.

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