Kinetic Behavior of Porcine Pancreatic Phospholipase A_2 on Zwitterionic and Negatively Charged Single-Chain Substrates†

Maarten G. van Oort, Ruud Dijkman, Jan D. R. Hille,‡ and Gerard H. de Haas*
Laboratory of Biochemistry, State University of Utrecht, Transitorium 3, University Centre "de Uithof", Padualaan 8, NL-3584 CH Utrecht, The Netherlands

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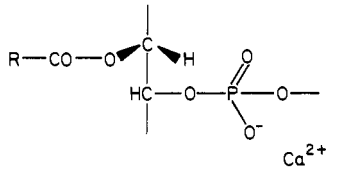
ABSTRACT: The kinetic properties of porcine pancreatic phospholipase A_2 were studied on a series of *n*-acylglycollecithins and *n*-acylglycol sulfates containing acyloxy or acylthio ester bonds at substrate concentrations below and above the critical micelle concentration. These single-chain detergents containing a primary (thio) ester bond are hydrolyzed rather slowly by the pancreatic enzyme, and maximal activity was found always for the *n*-octanoyl derivatives. The acylthio ester group is split 4–5 times faster than the corresponding acyloxy ester function. The kinetic behavior of the enzyme acting on zwitterionic glycollecithins or on anionic glycol sulfates is quite different and provides an explanation for the differences in pH optimum. Both for glycollecithins and for glycol sulfates, maximal enzyme activities are found in high molecular weight aggregates consisting of several enzyme molecules and detergent monomers. Their pathway of formation, however, is not the same.

Pancreatic phospholipase A_2 (EC 3.1.1.4) catalyzes the hydrolysis of the 2-acyl ester linkage in all common types of natural glycerophosphatides with an absolute requirement for Ca^{2+} ions. De Haas & van Deenen (1963) determined the minimal substrate requirements of this enzyme: an acyl ester bond has to be present in a position adjacent to the C–O–P linkage and the acyl group has to occupy a certain stereochemical configuration:



Besides its specific role in catalysis, Ca^{2+} most probably also functions as a bridge between the ionized phosphate group and Asp-49 in the active center of the enzyme (Verheij et al., 1980). Only this latter function can be assumed by Ba^{2+} or Sr^{2+}, which are pure competitive inhibitors of the enzyme.

In the past, it has been repeatedly demonstrated (van Deenen et al., 1962; de Haas & van Deenen, 1963; Hendrickson et al., 1981) that pancreatic phospholipases A_2, in contrast to most snake venom enzymes, possess a strong preference for negatively charged substrates. Long-chain neutral lecithins are hardly attacked by the pancreatic phospholipase unless deoxycholate or anionic phospholipids, such as phosphatidic acid or phosphatidylserine, are present. Under these conditions optimal enzyme activity is found at pH 9–9.5.

On the other hand, de Haas et al. (1971), working with short-chain lecithins, reported that the porcine enzyme effectively degraded micelles of these water-soluble substrates without the need of negative charge. A pH optimum of 6 was observed. In a kinetic study on medium-chain lecithins present as a monomolecular surface layer, Zografi et al. (1971) confirmed this slightly acidic pH optimum, whereas Verger et al. (1973), using the same technique, showed two pH optima for this enzyme: one at pH 6 and a second at pH 9. Taking into account the proposed reaction mechanism for pancreatic phospholipase (Verheij et al., 1980), k_{cat} is expected to be pH-independent between pH 6 and pH 9. This means that some of the previously reported "V_{max}" values may only be apparent, influenced by incomplete binding of the enzyme to differently charged interfaces. In order to clarify this confusion, a careful comparison of the kinetic behavior of the enzyme toward both negatively charged substrates and the corresponding zwitterionic derivatives is required. Taking into account the above-mentioned minimal substrate requirements, preference is given in this study to substrates containing one single acyl chain. Such single-chain substrates possess a high solubility in water, and even in the presence of the kinetically essential Ca^{2+} ions, optically clear monomeric and micellar solutions can be obtained. Direct binding studies between enzyme and substrates can be performed with spectroscopic techniques after replacement of Ca^{2+} by Sr^{2+} to avoid enzymatic breakdown. Up to now, the use of such single-chain detergents has been limited because of their extremely weak substrate properties (Slotboom et al., 1963; Bonsen et al., 1972). Recently, however, it has been shown that reliable kinetic results can be obtained with single-chain substrates after improvement of their substrate properties and of the assay conditions. Aarsman & van den Bosch (1979) reported that acyl*thio* ester bonds are effectively split by phospholipase A_2.

Their use allows a highly sensitive continuous assay of the enzyme by spectrophotometry. Moreover, Hille et al. (1983a,b) demonstrated that substitution of the phosphate moiety by a sulfate group results in an anionic center that is well-recognized by pancreatic phospholipase. This paper therefore deals with the kinetic properties of porcine pancreatic phospholipase A_2 on a series of glycollecithins[1] and glycol

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* Address correspondence to this author.
‡ Present address: Gist-Brocades N.V., NL-2600 MA Delft, The Netherlands.

[1] Abbreviations: PLA, porcine pancreatic phospholipase A_2; cmc, critical micelle concentration; ANS, 8-anilinonaphthalenesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); 4-PDS, di-4-pyridyl disulfide; HPTLC, high-performance thin-layer chromatography; IEP, isoelectronic point; glycollecithin, 2-*O*-acylglycol-1-phosphocholine; glycol sulfate, 2-*O*-acylglycol-1-sulfate; DPH, 1,6-diphenyl-1,3,5-hexatriene; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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sulfates possessing *O*-acyl and *S*-acyl ester bonds.

Materials and Methods

Porcine pancreatic prophospholipase A_2 was isolated from porcine pancreas and converted into active PLA by limited proteolysis as described by Nieuwenhuizen et al. (1974). Protein concentrations were determined from absorbance at 280 nm with an $E^{1\%}_{1cm}$ of 13.0. DTNB was obtained from Aldrich, and 4-PDS was a Sigma product. DPH was supplied by Janssen. ANS from K and K Laboratories was obtained as the sodium salt and converted into the magnesium salt by repeated crystallization from concentrated magnesium chloride solutions. All other chemicals were purchased from Merck (analytical grade).

Preparation of Substrates. The various monoacylglycols and 2-(monoacylthio)ethanol derivatives were prepared from glycol and 2-mercaptoethanol by established procedures (Aarsman et al., 1976; Hille et al., 1983b). These HPTLC-pure samples were converted into the phosphocholine derivatives as described by Eibl et al. (1967). The corresponding glycol sulfates were obtained as described by Hille et al. (1983b). Final purification of the glycollecithins was performed by silicic acid column chromatography and that of the glycol sulfates by crystallization of the sodium salts from 2-propanol. Spots on TLC plates were visualized by exposure to I_2 vapor or by spraying with 30% (v/v) sulfuric acid and charring (for the *O*-acyl-containing esters). For the *S*-acyl-containing compounds the more sensitive molybdate spray reagent was used (25 mL of concentrated H_2SO_4 + 500 mL of acetic acid containing 25 g of molybdatophosphoric acid). Compounds with free SH groups were detected as yellow spots after spraying with 5 mM DTNB in 0.5 M Tris-HCl, pH 7.5.

Critical Micelle Concentrations. Critical micelle concentrations for the substrates were determined in buffer solution either by the Wilhelmy plate method (Davies & Rideal, 1961) or by means of the soluble fluorescent probe ANS (in the absence of Ca^{2+}), as described by De Vendittis et al. (1981), and DPH (in the presence of Ca^{2+}), as described by Chattopadhyay et al. (1984). ANS was excited at 370 nm, and emission was recorded at 470 nm. For DPH the excitation and emission wavelengths were 358 and 430 nm, respectively.

cmc values for (*n*-decanoylthio)glycollecithin, *n*-decanoylglycollecithin, and *n*-dodecanoylglycollecithin are 1.4, 5.1, and 0.4 mM, respectively. Other cmc values are given in Table I and in the legend of Figure 1.

Kinetic Analysis. The hydrolysis of the thio ester substrates catalyzed by PLA was followed in a spectrophotometric assay and in a titrimetric assay. In the spectrophotometric assay, the appearance of SH groups was measured as described by Volwerk et al. (1979) in a Hitachi 150-20 spectrophotometer. Activities were calculated by using an E_{412} of 13 600 M^{-1} cm^{-1} for DTNB and an E_{324} of 19 800 M^{-1} cm^{-1} for 4-PDS.

The hydrolysis of the oxy ester substrates was followed by continuous titration of the liberated fatty acids as described by de Haas et al. (1971). However, the NaOH concentration was changed to 10 mM, and the experiments were done at 25 °C. Incidentally, a third kinetic analysis method described by Allgyer & Wells (1979) was used. In this spectrophotometric assay a pH indicator (phenolsulfonephthalein) was used to buffer the solution, and the rate of disappearance of the basic form of the indicator (upon liberation of fatty acids) was used to measure the rate of hydrolysis of the substrate. With this assay, only the hydrolysis of the neutral zwitterionic lecithins could be measured—the negatively charged glycol sulfates perturb the assay. The reaction cuvette contained 2.5 mL of substrate solution, 25 mM $CaCl_2$, and 1 mM indicator adjusted to pH 7.5. Absorbance was measured at 595 nm (a region where absorption by the acidic form is negligible) in a Shimadzu spectronic 200 spectrophotometer.

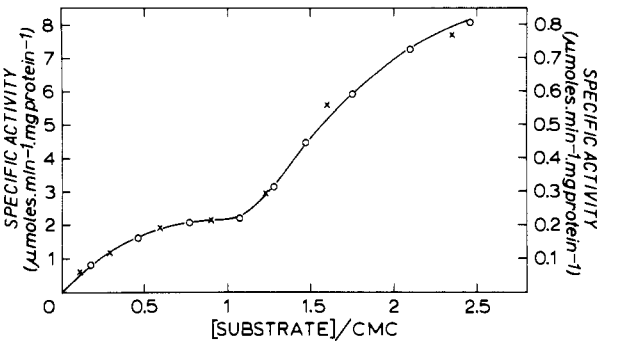


FIGURE 1: Specific activity of phospholipase A_2 at various substrate to cmc ratios for (*n*-octanoylthio)glycollecithin [(O) left ordinate] and *n*-octanoylglycollecithin [(×) right ordinate]. The cmc values for (*n*-octanoylthio)glycollecithin and *n*-octanoylglycollecithin are 19.8 and 59 mM, respectively. Conditions: 1 mM Pipes, 25 mM $CaCl_2$, pH 6.0, T = 25 °C. Note: different scales on both ordinates.

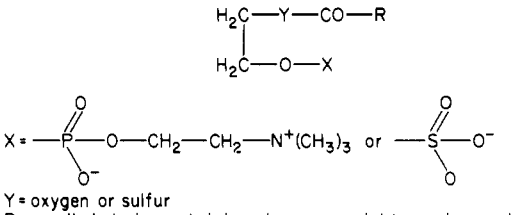
ANS Fluorescence. ANS fluorescence spectra were measured with a Perkin-Elmer LS-5 spectrofluorometer at 25 °C in 1-cm cells as described by Hille et al. (1983b) and by van Eyk et al. (1984).

Equilibrium Gel Filtration. A column (0.9 × 30 cm) was packed with Sephadex G-100 superfine (Pharmacia), equilibrated at 25 °C in 10 mM sodium acetate at pH 6.0 (10 mM borate at pH 9.0), 25 mM $SrCl_2$, and various concentrations of substrate. The column was used in an upward flow direction, and a constant amount of enzyme, dissolved in the equilibrium buffer, was passed through the column. From the elution volume, the molecular weight of the detergent–protein complex could be estimated after calibration of the column with a Boehringer protein Combithek, size II unit. For more details, see de Araujo et al. (1979).

Enzyme Inactivation. Enzyme inactivation by phenacyl bromide was carried out in 10 mM borate buffer, pH 7.75, at 25 °C according to Volwerk et al. (1974).

Results

The following glycol lipids have been investigated:



Under the experimental conditions (25 mM Ca^{2+}) the sulfates possess cmc values that are rather similar to those of the corresponding glycollecithins. In contrast to the zwitterionic glycollecithins, the cmc values of the anionic sulfates are very sensitive to the ionic strength of the solution. In the absence of Ca^{2+} ions, the cmc values of the glycol sulfates are about 2 times higher. Substitution of the *O*-acyl group by the thio ester moiety results in a 4–5-fold decrease of the cmc due to the more hydrophobic character of sulfur. Figures 1 and 2 show the kinetic properties of phospholipase A_2 on (thio) glycollecithins (Figure 1) and (thio) glycol sulfates (Figure 2) at one alkyl chain length (R = C_7H_{15}).

Table I: Maximal Activity [μmol min^{-1} (mg of Protein)$^{-1}$] of Porcine Pancreatic Phospholipase A_2 at pH 6 and pH 9 on *O*-Acylglycol Sulfates and *S*-Acylglycol Sulfates[a]

	O-acylglycol sulfates			*S*-acylglycol sulfates		
	maximal activity			maximal activity		
alkyl chain length	pH 6.0[b]	pH 9.0[c]	cmc (mM)	pH 6.0[b]	pH 9.0[c]	cmc (mM)
6	0.3 (0.21)	~2[d]	~140	1.3 (0.14)	~10	~35
7	1.4 (0.21)	5	49	6.7 (0.12)	20	11
8	1 (0.20)	4	15.6	6.0 (0.12)	17.5	3.5
9	0.3 (0.19)	2.5	5.4	1.5 (0.13)	10.3	1.0

[a] Conditions: (pH 6.0) 1 mM Pipes, 25 mM Ca^{2+}, 25 °C; (pH 9.0) 1 mM borate, 25 mM Ca^{2+}, 25 °C. [b] Values in parentheses are the ratio of substrate concentration to cmc at which maximal activity is reached, cf. Figure 2. [c] Maximal activities at pH 9.0 were measured at substrate concentration well above the cmc, cf. Figure 5. [d] No reliable values could be obtained because of the very high cmc of these detergents. The concentrated detergent solutions required in kinetic studies show high blank values at pH 9.0 because of nonenzymatic hydrolysis.

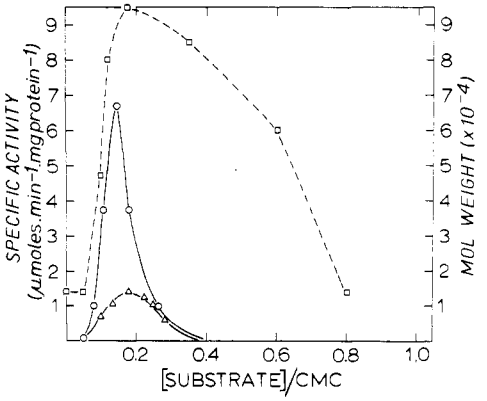


FIGURE 2: Specific activity of phospholipase A_2 at various substrate to cmc ratios for (*n*-octanoylthio)glycol sulfate (○) and for *n*-octanoylglycol sulfate (△). Conditions: 1 mM Pipes, 25 mM $CaCl_2$, pH 6.0, T = 25 °C. The dotted line represents the molecular weight of the phospholipase A_2–substrate complex as a function of the glycol sulfate concentration [right ordinate (□)]. Conditions: 10 mM Pipes buffer, 25 mM $SrCl_2$, pH 6.0, T = 25 °C.

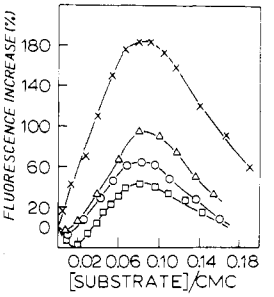


FIGURE 3: ANS fluorescence at various substrate to cmc ratios in the presence of 7.7 μM phospholipase A_2. Conditions: 10 mM Pipes buffer, 25 mM $SrCl_2$, T = 25 °C, [ANS] = 16.1 μM. (□) (*n*-heptanoylthio)glycol sulfate, R = six; (○) (*n*-octanoylthio)glycol sulfate, R = seven; (△) (*n*-nonanoylthio)glycol sulfate, R = eight; (×) (*n*-decanoylthio)glycol sulfate, R = nine.

Very different behavior is observed for the zwitterionic glycollecithins and the anionic sulfates at pH 6.0. First note the order of magnitude differences in the ordinates in Figure 1 for the two glycollecithins. Below their cmc, PLA acts on monomeric substrate molecules in a 1:1 molar ratio, while a normal Michaelis–Menten-type saturation curve is obtained with maximal velocity around the cmc of the substrate. Above the cmc, large spherical micelles are known to form (de Araujo et al., 1979). The enzyme recognizes the detergent–water interface, and upon interaction, high molecular weight lipid–protein aggregates are formed (Hille et al., 1981; Donné-Op den Kelder et al., 1981). In these complexes, PLA displays much higher enzymatic activity. V_{max} values are reached at substrate concentrations several times higher than the cmc, indicating a rather weak affinity of the enzyme for a micellar surface. This kinetic behavior is rather pH-independent, since curves similar to those of Figure 1 were found between pH 6 and pH 9. It is evident that the substitution of the oxygen ester in the glycollecithin by a thio ester group considerably improves the substrate properties.

As illustrated in Figure 2, the enzyme displays very different kinetics at pH 6 in the presence of the negatively charged (thio) glycol sulfates, since a sharp increase in enzyme activity occurs at substrate concentrations far below the cmc. Maximal activity is found at about 0.15 × cmc, followed by a rapid decrease at higher lipid concentrations. Concomitant with the rise in PLA activity as seen in Figure 2, there is a significant increase in the molecular weight of the enzyme–detergent complex, reaching a maximal value of 95 000 at about 0.15 × cmc. At higher detergent concentrations, the premicellar aggregates seem to fall apart since already before the cmc the enzyme is again present in monomeric form.[2] Enzyme activity is hardly detectable at higher detergent concentrations. Similar curves were obtained for the other (thio) glycol sulfates. As shown in Table I, independent of the acyl chain length, the submicellar optima at pH 6 were found around 0.13 × cmc for the *S*-acyl glycol sulfates while the *O*-acyl derivatives possess maximal activity at about 0.20 × cmc. Most probably, this small shift is related to the higher PLA concentrations required in the determination of the specific activities of the *O*-acyl derivatives.

In order to study the interaction of PLA with various *S*-acyl glycol sulfates, the fluorescent probe 8-anilinonaphthalenesulfonic acid was used. This probe is nonfluorescent in water, but becomes highly fluorescent when bound to macromolecules or aggregated structures. Concomitant with the rise in fluorescence quantum yield, a blue shift in the wavelength at which maximum fluorescence occurs can be observed. Therefore, this probe was used to obtain information on the origin and breakdown of high molecular weight aggregates when PLA interacts with anionic lipids at submicellar substrate concentrations. A mixture of PLA + ANS was titrated with increasing amounts of the thioglycol sulfates, and ANS fluorescence was recorded as a function of the detergent concentration (see Figure 3). In order to allow comparison with the kinetic results, fluorescence measurements were conducted with Sr^{2+} instead of Ca^{2+} to avoid enzymatic breakdown. Bell-shaped curves were obtained with all thioglycol sulfates, and maximal fluorescence quantum yield was found at 0.08 × cmc. An increase in maximal fluorescence with the *O*-acylglycol sulfates also takes place at 0.08 × cmc, amounting to 47% (for the R = 7 analogue), 80% (for the R = 8 analogue), and 140% (for the R = 9 derivative).

[2] The slower decay of the "molecular weight" as compared to the descending part of the specific activity curve is most probably caused by the quite different amounts of enzyme required in these measurements: ~0.5 μM in the kinetic studies vs. >30 μM in the molecular weight determinations.

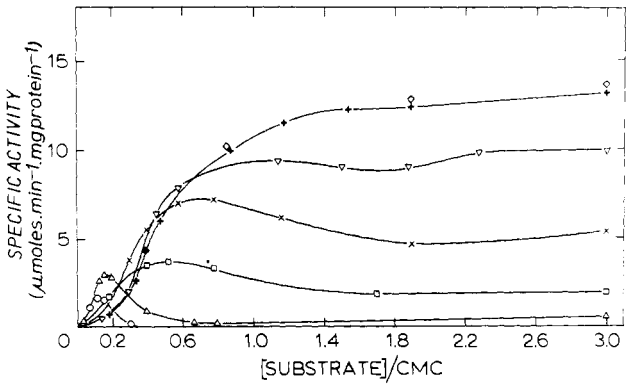


FIGURE 4: Specific activity of porcine pancreatic phospholipase A_2 as function of (*n*-decanoylthio)glycol sulfate concentration between pH 6 and pH 10. Conditions: (pH 6.0) 1 mM Pipes, 25 mM $CaCl_2$; (pH 7.0) 1 mM Hepes, 25 mM $CaCl_2$; (pH 8.0–10.0) 1 mM borate, 25 mM $CaCl_2$; T = 25 °C. (○) pH 6.0; (△) pH 7.0; (□) pH 8.0; (×) pH 8.5; (▽) pH 9.0; (+) pH 9.5; (◇) pH 10.0.

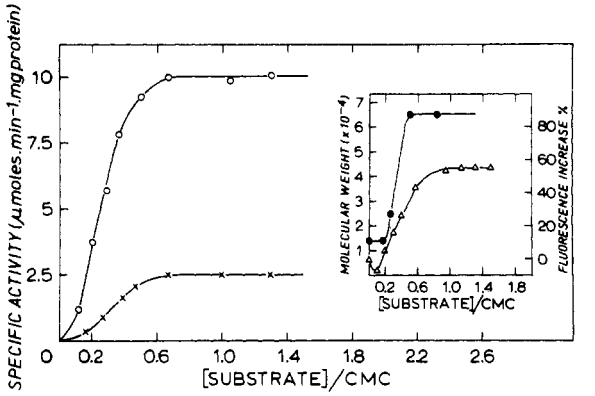


FIGURE 5: Specific activity as function of substrate cmc ratio at pH 9.0: (upper curve) (*n*-decanoylthio)glycol sulfate (○); (lower curve) *n*-decanoylglycol sulfate (×). Conditions: 1 mM borate buffer, 25 mM $CaCl_2$, pH 9.0, T = 25 °C. (Inset) Molecular weight of the phospholipase A_2–substrate complex as function of (*n*-decanoylthio)glycol sulfate concentration [left ordinate (●)]. The right ordinate gives the ANS fluorescence increase as function of (*n*-decanoylthio)glycol sulfate concentration (△). Conditions: 10 mM borate buffer, 25 mM $SrCl_2$, pH 9.0, T = 25 °C, [ANS] = 16.1 μM.

These fluorescence curves strongly resemble the bell-shaped activity profiles also seen in Figure 2, except that the kinetic maxima are found at about 0.15 × cmc. The shift of the fluorescence maxima to a lower detergent concentration can be quantitatively explained by the substitution of Ca^{2+} by Sr^{2+} The gradual substitution of Ca^{2+} by Sr^{2+} sharply lowers enzyme activity and at the same time the substrate concentration, giving maximal enzyme activity shifts from 0.14 × cmc to 0.08 × cmc. Although Sr^{2+}, in the absence of substrate, binds to PLA with the same affinity as Ca^{2+} and has been shown to be a pure competitive inhibitor for Ca^{2+} in kinetic studies (Pieterson et al., 1974), it can be concluded that in the ternary E–M^{2+}–detergent complex Sr^{2+} is bound more firmly.

The small decrease of the fluorescence quantum yield upon addition of very low concentrations of (thio) glycol sulfates as shown in Figure 3 indicates that ANS binds to the enzyme but is rapidly released in the presence of small amounts of detergent. Apparently, the detergent monomers compete with ANS for the same binding site on the enzyme and affinity increases with increasing chain length of the detergent. The dissociation constant of the phospholipase A_2–ANS complex was determined as described by van Eyk et al. (1984): A low ANS concentration was titrated with increasing amounts of enzyme, and the rise in ANS fluorescence quantum yield was plotted as function of the enzyme concentration (results not shown). From the saturation curve the fluorescence increase per mole of ANS bound to the enzyme can be derived. By use of this value and the results of a second titration in which fluorescence is measured for a constant enzyme concentration at increasing ANS concentrations, a Scatchard plot can be constructed. Both titrations yield a K_D value of 200 μM and a stoichiometry of two ANS per enzyme molecule.

In order to get more information on the binding loci of ANS on the enzyme surface, use was made of the irreversible active site directed inhibitor *p*-bromophenacyl bromide (Volwerk et al., 1974). This compound has been shown to react covalently with the N_1 of His-48 located in the active center of the enzyme with complete loss of catalytic properties (Verheij et al., 1980). The inhibition reaction shows pseudo-first-order kinetics with a $t_{1/2}$ of 5 min (10 mM borate, pH 7.75). In the presence of 770 μM ANS, considerable protection is observed, resulting in longer $t_{1/2}$ values. This indicates that at least one of the ANS molecules binds in the active center close to His-48. This confirms similar findings for various venom PLA's (Yang & King, 1980a,b; Barden et al., 1980; van Eyk et al., 1984).

It must be mentioned that, in contrast to the behavior of the glycollecithins, the activity profiles of the anionic sulfates (as shown in Figure 2) are strongly pH-dependent. Figure 4 illustrates also that when the pH is increased, the sharp optimum in enzyme activity, found at pH 6 and 7, gradually broadens and shifts to higher substrate concentration. At pH ≥9, sigmoidal saturation curves are obtained that reach maximal activity around the cmc of the substrate, and the hydrolysis rates remain roughly constant up to substrate concentrations far above the cmc. This is seen more clearly in Figure 5, comparing the specific activity of PLA acting on *n*-decanoylglycol sulfate and the corresponding thio ester analogue at pH 9. Both substrates show a similar saturation curve, and again, the thio ester containing detergent is hydrolyzed much faster than the *O*-acyl sulfate.

The inset of Figure 5 demonstrates that at pH 9 high molecular weight enzyme–detergent complexes are formed. These aggregates (M_r ~65 000) are smaller than those formed at pH 6, and they do not seem to fall apart at higher detergent concentrations. The different composition of these complexes as compared with the premicellar aggregates formed at pH 6 is indicated also by the ANS fluorescence. The maximal increase in fluorescence quantum yield when ANS is incorporated in the alkaline complexes amounts to 55% whereas in the slightly acidic aggregates more than 180% increase is observed (Figure 3).

In Table I the specific activities of the various (thio) glycol sulfates at pH 6 and 9 are summarized. Both for the *O*-acyl- and for the *S*-acylglycol sulfates highest enzymatic activity is found for the octanoyl and nonanoyl derivatives (R = seven or eight), and a considerable fall in specific activity occurs with the shorter *n*-heptanoyl sulfates (R = six) and the longer *n*-decanoyl compounds (R = nine). Both at pH 6 and at pH 9 the thio ester group is cleaved at a 4–5 times higher rate than the corresponding oxygen ester.

Finally, Figure 6 shows the dramatic difference between the anionic glycol sulfates and the zwitterionic glycollecithins in activity measurements at substrate concentrations *above* the cmc.

Discussion

This study deals with the substrate properties of a series of monoacyl glycol lipids for pancreatic phospholipase A_2. Structure variations were introduced both at the polar head

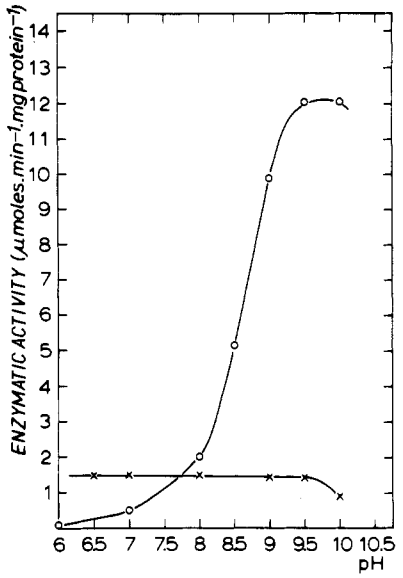


FIGURE 6: pH dependence of phospholipase A_2 acting on (*n*-decanoylthio)glycol sulfate (O) and *n*-decanoylglycollecithin (×). Conditions: (pH 6.0) 1 mM Pipes, 25 mM $CaCl_2$; (pH 7.0) 1 mM Hepes, 25 mM $CaCl_2$; (pH 8.0–10.0) 1 mM borate buffer, 25 mM $CaCl_2$; T = 25 °C. Note: substrate concentration 3 × cmc.

group and the acyl ester function. From the results presented in this paper, it is clear that the glycol lipids with one single acyl chain and an ester or thio ester bond possess weak substrate properties. Notwithstanding the fact that they contain the minimal structural elements required by PLA, V_{max} values under optimal conditions could not be raised above 10–20 μmol min^{-1} (mg of protein)$^{-1}$. This low catalytic efficiency does not seem to be related to the tendency to form micelles alone, since both the sulfate- and phosphocholine-containing detergents display optimal enzyme activity at an acyl chain length of 8 carbon atoms. Similar behavior has been reported before for the *di*acyl-*sn*-glycero-3-phosphocholines (de Haas et al., 1971); dioctanoyllecithin is degraded >10 times faster than the diheptanoyl- and dinonanoyllecithins.

Possibly, the octanoyl chain optimally fits the active center of the enzyme. This observation should be kept in mind when optimal reversible inhibitors are designed.

Table I shows that substitution of the *O*-acyl ester group by a thio ester function results in a 4–5 times higher hydrolysis rate for all glycol sulfates. From Figure 1 it is evident also that the substrate properties of the glycollecithins considerably improve with this substitution. The preference of PLA for a thio ester linkage over the corresponding oxygen ester has been reported before (Aarsman & van den Bosch, 1979; Volwerk et al., 1979; Hendrickson et al., 1983). A similar increase of k_{cat} in the hydrolysis of thio esters as compared to the oxygen esters has been observed for several Ser-esterases (Aarsman & van den Bosch, 1979; Hirohara et al., 1974; Ahmed et al., 1984). It has been explained by the better leaving properties of SH compounds in the decomposition of the tetrahedral intermediate. However, see Discussion in the accompanying paper (van Oort et al., 1985b).

Comparison of Figures 1 and 2 indicates that substitution of the phosphocholine group by the negatively charged sulfate for the same alkyl chain length (R = seven) results in rather similar maximal hydrolysis rates at pH 6.0. It should be realized, however, that such a comparison is perilous because of the different kinetics observed for the hydrolysis of the neutral glycollecithins and the anionic glycol sulfates. The substrate concentrations at which maximal velocities are reached, for example, are widely different: ~0.15 × cmc for the sulfates and (3–4) × cmc for the glycollecithins. Although with both classes of detergent maximal PLA activity is found in large enzyme–detergent aggregates with a similar molecular weight of about 90 000, the composition of the complexes is different. Model studies with *n*-alkylphosphocholines which are nondegradable substrate analogues, very similar to glycollecithins, showed that the neutral lipid–protein aggregates consist of 2 enzyme molecules and about 70 detergent monomers (de Araujo et al., 1979; Hille et al., 1981; Donné-Op den Kelder et al., 1981). On the other hand, the premicellar aggregates of PLA, with the anionic glycol sulfates, contain 6 enzyme molecules and about 40 detergent monomers (Hille et al., 1983b). The much higher detergent/enzyme ratio in the phosphocholine-containing complexes as compared to anionic aggregates reflects the different pathways along which the complexes are formed. Pancreatic PLA does not aggregate in glycollecithin solutions below the cmc (Volwerk et al., 1979), while high molecular weight lipid–enzyme complexes are formed only by interaction of the enzyme with preexisting detergent micelles (de Araujo et al., 1979). This is in sharp contrast to the behavior of the enzyme in dilute glycol sulfate solutions. Already at substrate concentrations far below the cmc, a highly cooperative aggregation process starts; complexes are formed containing six enzyme molecules and about seven detergent monomers per enzyme molecule (Hille et al., 1983b). Notwithstanding the fact that both types of complexes possess similar molecular weights and strongly activate the catalytic properties of PLA, the different composition is indicated also by their respective stabilities. In the phosphocholine-containing aggregates the stoichiometry of the lipid–protein complex remains constant over the pH range 6–9 (Donné-Op den Kelder et al., 1981). On the other hand, the premicellar enzyme–sulfate complexes can exist at neutral pH in a very narrow detergent concentration range only, and the aggregates seem to fall apart at slightly higher or lower substrate concentration (Figure 2). At higher pH values, however, the premicellar PLA–sulfate complexes became progressively more resistant to excess detergent, and at pH values ≥9, molecular weight determinations and ANS fluorescence (Figure 5) point to enzyme–detergent aggregates with a molecular weight of about 65 000. These complexes possess high enzymatic activity and are stable also above the cmc of the detergent. These properties provide an explanation for the seemingly very different kinetics of PLA acting on micellar solutions of glycollecithins and sulfates as shown in Figure 6.

It has to be noted that similar behavior was recently reported by van Eyk et al. (1983) for a venom PLA acting on submicellar concentrations of a neutral *lecithin* at pH 8. In the presence of Ca^{2+}, lipid–protein aggregates with a M_r of 65 000 were formed at substrate concentrations below the cmc. These complexes consist of 4 enzyme molecules and about 30 detergent monomers and display extremely high enzymatic activity. They remain stable also above the cmc of the substrate.

Taken together, our observations strongly suggest that, at least for the lipolytic enzymes of the A_2 type, high catalytic efficiency is intimately related to the formation of high molecular weight aggregates containing several protein molecules and at least six or seven substrate monomers per enzyme molecule. The possible significance of protein self-association with regard to catalysis has been demonstrated most clearly by Mishin et al. (1982) for pancreatic lipase.

Finally, two important questions remain to be answered: What is the molecular basis for high PLA activity in aggregates containing several enzyme and substrate molecules? Why do these premicellar complexes appear to fall apart at

slightly higher detergent concentrations in neutral solution and not under more alkaline conditions?

The first question is the central question for all lipolytic enzymes in general. They all act preferentially on organized lipid–water interfaces, and so far, no generally accepted theory for interfacial activation has been provided (Slotboom et al., 1982; Volwerk & de Haas, 1982; Dennis, 1983; Verger, 1984). In the premicellar aggregates the detergent monomers could provide a lipid–water interface favorable for enzymatic attack. On the other hand, the presence of several PLA molecules indicates that protein–protein interactions also might be playing an important role.

In order to answer the second question, we must know how these aggregates are formed. The high-resolution X-ray structures of the bovine and porcine PLA (Dijkstra et al., 1978, 1981a,b, 1983) show that in these pancreatic enzymes the active site is surrounded by a collar of hydrophobic amino acid side chains located at the surface of the protein. This is a rather unusual situation in globular proteins that could lead to an enhanced tendency to aggregate in aqueous solutions. Many PLA's from snake venom origin are indeed prone to dimer formation. For a review, see Dennis (1983). The reason that the pancreatic phospholipases in aqueous solution remain molecularly dispersed most probably has to be attributed to the presence of several positively charged side chains of Lys and Arg residues in the lipid binding domain. Hille et al. (1983a,b) demonstrated that in dilute solutions of negatively charged detergents the pancreatic PLA binds one or two anionic monomers to the lipid binding domain with high affinity. The neutralization of the positive surface charges in the lipid binding domain of the enzyme combined with the increased surface hydrophobicity would be expected to lead to a much higher tendency to aggregate, especially at the isoelectric point (pH 6) of the enzyme. This protein aggregation would be accompanied and stabilized by a concomitant gathering of detergent molecules at lipid concentrations far below the cmc and results in enzyme–substrate complexes with a M_r of 95000 consisting of 6 PLA molecules and about 40 detergent monomers. At this moment, we can only speculate on the molecular structure of these high molecular weight complexes. Perhaps they are premicellar lipid aggregates stabilized by an annulus of protein molecules or reversed detergent micelles stabilizing protein aggregates. In such relatively small lipid–protein aggregates, however, direct protein–protein interactions must be present, and one can imagine that the nonspecific binding of additional anionic lipid molecules to the protein surface at higher detergent concentration will lead to increased protein–protein repulsion and consequently to a breakup of the aggregates. At pH values far above the IEP of the enzyme, the negative surface charge of the protein could counteract the nonspecific binding of anionic detergent molecules. Aggregation would then start at higher detergent concentrations, and the resulting complexes would contain less protein and detergent. Apparently, the repulsive forces between the protein molecules would then be less in these smaller aggregates, and they would remain stable above the cmc.

Registry No. $CH_3(CH_2)_5CO_2(CH_2)_2OSO_3H$, 99165-75-0; $CH_3(CH_2)_6CO_2(CH_2)_2OSO_3H$, 99165-76-1; $CH_3(CH_2)_7CO_2(CH_2)_2OSO_3H$, 99165-77-2; $CH_3(CH_2)_8CO_2(CH_2)_2OSO_3H$, 99165-78-3; $CH_3(CH_2)_5C(O)S(CH_2)_2OSO_3H$, 99165-79-4; $CH_3(CH_2)_6C(O)S(CH_2)_2OSO_3H$, 99165-80-7; $CH_3(CH_2)_7C(O)S(CH_2)_2OSO_3H$, 99165-81-8; $CH_3(CH_2)_8C(O)S(CH_2)_2OSO_3H$, 99165-82-9; $CH_3(CH_2)_5CO_2(CH_2)_2OPO_3^-(CH_2)_2NMe_3^+$, 99165-83-0; $CH_3(CH_2)_6CO_2(CH_2)_2OPO_3^-(CH_2)_2NMe_3^+$, 41107-76-0; $CH_3(CH_2)_7CO_2(CH_2)_2OPO_3^-(CH_2)_2NMe_3^+$, 99165-84-1; $CH_3(CH_2)_8CO_2(CH_2)_2OPO_3^-(CH_2)_2NMe_3^+$, 99165-85-2; $CH_3(CH_2)_5C(O)S(CH_2)_2OPO_3^-(CH_2)_2NMe_3^+$, 99165-86-3; $CH_3(CH_2)_6C(O)S(CH_2)_2OPO_3^-(CH_2)_2NMe_3^+$, 99165-87-4; $CH_3(CH_2)_7C(O)S(CH_2)_2OPO_3^-(CH_2)_2NMe_3^+$, 99165-88-5; $CH_3(CH_2)_8C(O)S(CH_2)_2OPO_3^-(CH_2)_2NMe_3^+$, 69339-95-3; phospholipase A_2, 9001-84-7.

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Kinetic Behavior of Porcine Pancreatic Phospholipase A_2 on Zwitterionic and Negatively Charged Double-Chain Substrates†

Maarten G. van Oort, Ruud Dijkman, Jan D. R. Hille,‡ and Gerard H. de Haas*
Laboratory of Biochemistry, State University of Utrecht, Transitorium 3, University Centre "de Uithof", Padualaan 8, NL-3584 CH Utrecht, The Netherlands
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ABSTRACT: A number of isomeric diacylglycerophosphocholines and diacylglycero sulfates containing *O*-acyl and/or *S*-acyl ester bonds were investigated as substrates for porcine pancreatic phospholipase A_2 and its zymogen. A comparison is made with the kinetic properties of the enzyme toward the corresponding glycol detergents previously described [van Oort, M. G., Dijkman, R., Hille, J. D. R., & de Haas, G. H. (1985) *Biochemistry* (preceding paper in this issue)]. Hydrolysis of the secondary ester bond in the 1,2-diacylglycero-3-type lipids proceeds much faster than the splitting of the primary ester function present in the isomeric 1,3-diacylglycerol and 1-acylglycol derivatives. In sharp contrast to the glycol detergents, the substitution of the cleavable oxygen ester by a thio ester bond in the glycerol lipids results in 5 times lower k_{cat} values. At alkaline pH and above the critical micelle concentration, the anionic sulfates are much better substrates than the corresponding phosphocholine-containing detergents. At very low detergent concentrations, below the critical micelle concentration, the anionic sulfates induce protein aggregation such that phospholipase A_2, as well as its zymogen, is present in high molecular weight complexes containing several protein molecules. In these aggregates, protein–protein and/or lipid–protein interactions strongly activate phospholipase but not the zymogen.

The preceding paper (van Oort et al., 1985) dealt with the kinetic behavior of porcine pancreatic phospholipase A_2 (PLA)[1] acting on glycollecithins and glycol sulfates (C_2 detergents), containing one single acyl chain in a primary ester or thio ester bond. Highest specific activities were found both for the zwitterionic and for the anionic substrates in lipid–protein aggregates containing several enzyme molecules and a number of detergent monomers. It should be stated, however, that the maximal specific activities that can be reached with the single-chain glycol derivatives are low, in the order of 1–20 μmol min^{-1} (mg of protein)$^{-1}$. The natural substrates for this enzyme are 1,2-diacylglycerol derivatives (C_3 detergents) in which a secondary ester group is split. Therefore, it seemed of interest to include these lipids as well to see whether or not the kinetic anomalies observed with the C_2-detergents were caused by the presence of a single acyl chain. In addition, we studied the substrate properties of the isomeric 1,3-diacylglycerol lipids, which are intermediate between glycol lipids and 1,2-diacylglycerol derivatives. They and the glycol

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* Address correspondence to this author.
‡ Present address: Gist-Brocades N.V., NL-2600 MA Delft, The Netherlands.

[1] Abbreviations: PLA, pancreatic phospholipase A_2; proPLA, pancreatic prophospholipase A_2; C_2 detergents, *n*-acyl- or (*n*-acylthio)-glycolphosphocholines or *n*-acyl- or (*n*-acylthio)glycol sulfates; C_3 detergents, diacyl- or bis(acylthio)glycerophosphocholines or diacyl- or bis(acylthio)glycero sulfates. All other abbreviations used in this study have been compiled in the preceding paper (van Oort et al., 1985).

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