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

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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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[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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detergents both contain cleavable primary ester bonds. They also resemble the natural 1,2-diacylglycerol compounds because of their two acyl chains.

Materials and Methods

Most of the materials and methods used in this study have been described in the preceding paper (van Oort et al., 1985).

Preparation of Substrates. All C_3 detergents investigated in this study were prepared from the corresponding "diglycerides". *rac*-1,2-Diacylglycerol and *rac*-1,3-diacylglycerol were obtained by acylation of 3-benzylglycerol and 2-benzylglycerol, respectively, followed by catalytic hydrogenolysis with Pd/H_2. For the corresponding thioacyl derivatives, *rac*-3-mercapto-1,2-propanediol, 1,3-dimercapto-2-propanol, and *rac*-1,2-dimercapto-3-propanol (Janssen, Belgium) were acylated and freed from undesired isomers by fractional crystallization at low temperature. As an example, the synthesis of *rac*-1,3-diheptanoyl-3-mercapto-1,2-propanediol is described.

A total of 51 g (0.47 mol) of *rac*-3-mercapto-1,2-propanediol and 33.5 g of dry pyridine was dissolved in 300 mL of anhydrous chloroform. After this was cooled to −20 °C, 1.8 mol (126 g) of heptanoyl chloride, dissolved in 100 mL of dry chloroform, was added dropwise under stirring, keeping the temperature of the reaction mixture below −10 °C. After addition of the acyl chloride, the reaction mixture was kept at 0 °C for 1 h and subsequently washed with ice-cold 0.5 N HCl and water. Thin-layer chromatography on silicic acid in ether–hexane, 1:1 v/v, revealed one major spot of the desired 1,3-diglyceride (R_f 0.54) and two minor spots of the monoacylated derivatives[2] (R_f 0.13 and 0.43). The chloroform solution was dried over anhydrous Na_2SO_4 and evaporated to dryness in vacuo. The residue, a colorless oil, was dissolved in heptane and extracted several times with MeOH–H_2O, 90:10 v/v. TLC demonstrated that the most polar monoacylated derivative (R_f 0.13) was quantitatively extracted from the heptane phase.[3] After evaporation of the heptane, the residue was dissolved in dry acetone and crystallized at −60 °C. Pure *rac*-1,3-diheptanoyl-3-mercapto-1,2-propanediol, free of isomers, was obtained as colorless crystals melting below −10 °C in a yield of 30%.

rac-1,2-(Diheptylcarbamoyl)glycerol was synthesized according to Gupta & Bali (1981).

The above "diglycerides" were converted into *rac*-lecithins and diacylglycero sulfates by established procedures [see the preceding paper (van Oort et al., 1985)]. The end products were purified to homogeneity by silica column chromatography (for lecithins) and repeated crystallization from 2-propanol (for the diacylglycero sulfates).

In a number of cases the racemic C_3 detergents were converted into pure enantiomeric compounds by preparative PLA hydrolysis. With catalytic enzyme concentrations, hydrolysis stops at 50%, yielding the 2,3-diacyl-*sn*-glycero-1-type and 1-acyl-*sn*-glycero-3-type derivatives. After column chromatographic separation the latter lyso derivatives were converted into the 1,2-diacyl-*sn*-glycero lipids by chemical acylation.

Ultraviolet Absorbance Difference Spectroscopy. Difference spectra were recorded on an Aminco Model DW 2-a spectrophotometer equipped with a Midan data analyzer, coupled to an Apple II desk-top computer. Transferred spectra were stored on floppy disks. Measurements were carried out as described previously by de Araujo et al. (1979) and Hille et al. (1981).

Equilibrium Dialysis. Equilibrium dialysis was carried out as described in an earlier paper (Hille et al., 1983a).

Enzyme Inactivation Procedure. Rates of inactivation of PLA by phenacyl bromide were determined in 10 mM Hepes buffer containing 10 mM EDTA at 30 °C and pH 7.3. The reaction was initiated by the addition of 0.7 mg of phenacyl bromide dissolved in 20 μL of 2-propanol to 1 mL of buffer containing 0.5–1.0 mg of protein. This gave a 50–100-fold molar excess of reagent over protein. Inactivation of PLA essentially follows first-order kinetics, and therefore, inactivation rates can be measured conveniently by using a semilogarithmic plot of residual activity vs. time. This yields a straight line from which the halftime ($t_{1/2}$) can be determined. When a linear semilogarithmic plot is also observed in the presence of protecting substrate analogues, the method developed by Scrutton & Utter (1965) can be adopted to study the effect of protecting ligands. With a protecting ligand L, the following equation is valid:

$$t_{1/2}^0/t_{1/2} = \frac{k_2}{k_1} + K_L\frac{1 - (t_{1/2}^0/t_{1/2})}{[L]}$$

where $t_{1/2}^0$ and $t_{1/2}$ are the observed halftimes for the inactivation in the absence and presence of L and k_1 and k_2 are the rate constants for the inactivation of the free enzyme and the enzyme–ligand complex, respectively. K_L is the dissociation constant for the enzyme–ligand complex, and [L] is the equilibrium concentration of the protecting ligand.

Table I: Maximal Activity [μmol min^{-1} (mg of Protein)$^{-1}$] of Pancreatic PLA as a Function of Acyl Chain Length of the Substrate[a]

substrate	maximal activity [μmol min^{-1} (mg of protein)$^{-1}$]
1,2-dihexanoyl-*sn*-glycero-3-phosphocholine	45
1,2-diheptanoyl-*sn*-glycero-3-phosphocholine	103
1,2-dioctanoyl-*sn*-glycero-3-phosphocholine	551
1,2-dinonanoyl-*sn*-glycero-3-phosphocholine	107
1,2-diheptanoyl-*sn*-glycero 3-sulfate	904
1,2-dioctanoyl-*sn*-glycero 3-sulfate	5949
1,2-dinonanoyl-*sn*-glycero 3-sulfate	1714

[a] Conditions: 1 mM borate, pH 9,0, 25 mM $CaCl_2$, T = 25 °C. Substrate concentrations used were all well above the cmc of the lipid.

Results

Table I demonstrates that at pH 9.0 the anionic diacylglycero sulfates have much better substrate properties for the pancreatic enzyme than the zwitterionic diacylglycerophosphocholines. In addition, these results confirm the preference of the enzyme for acyl chains containing eight carbon atoms as was noted previously for the C_2 detergents described in the foregoing paper.

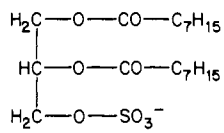
In order to find out whether the enzyme follows a similar reaction mechanism with C_2 and C_3 substrates, direct binding studies were required. Such studies can be done with a true substrate in the presence of Sr^{2+} instead of Ca^{2+} in order to prevent hydrolysis. If the specific action of Ca^{2+} is to be

[2] Note the remarkable difference in R_f values for the *O*-acyl ester (0.43) containing a free SH group and the isomeric *S*-acyl ester (0.13) with a free OH function.

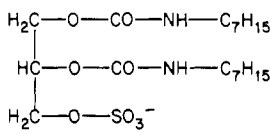
[3] Notwithstanding the very mild treatment, the heptane phase now contains a minor spot (R_f 0.77) reacting positively on free SH and that was not present in the original acylation mixture. This compound turned out to be 1,2-diheptanoyl-3-mercapto-1,2-propanediol, an isomerization product of the desired 1,3-diacyl derivative. Partial glycerides containing a free OH group adjacent to a thio ester bond are known (Jencks, 1969) to be very labile, and they isomerize easily into derivatives with an *O*-acyl ester linkage and a free SH group. Note again the considerable difference in R_f values for the isomeric 1,3- (R_f 0.54) and 1,2-"diglycerides" (R_f 0.77), which contain a free OH or a free SH group, respectively.

studied, the real substrate can be replaced by a nondegradable substrate analogue. The analogue should possess pure competitive inhibition properties and preferably have a K_i similar to the enzyme–substrate dissociation constant K_S. The enantiomeric *sn*-1-glycerophospholipids (D-α isomers) fullfil these requirements in a nearly ideal way. Acting on mixtures of stereoisomeric lecithins, in which the sum of the concentrations of the components is kept constant ([*sn*-3] + [*sn*-1] = constant), the enzyme shows a linear proportional relationship between specific activity and the percent *sn*-3 isomer in the *sn*-1 + *sn*-3 mixture (Bonsen et al., 1972). Such a linear relationship is observed for the diacylglycerophosphocholines and the diacylglycero sulfates independently of the fatty acyl chain length (results not shown). The fact that this behavior is independent of the *total* ([*sn*-1] + [*sn*-3]) detergent concentrations, both above and below the cmc of the lipid, proves that not only the apparent binding constants between enzyme and micellar surface but also the affinity to the catalytic site are identical for *sn*-1 and *sn*-3 monomers.

Direct binding studies with the anionic diacylglycero sulfates are plagued, however, by two major problems: Although the short-chain analogues are highly water-soluble in the absence of divalent cations, they readily precipitate in the presence of Ca^{2+}, and even more with Sr^{2+}. This property imposes serious limitations in our choice of binding techniques. A second difficulty with the C_3 sulfates is the extreme efficiency with which the enzyme acts upon these substrates; e.g., under optimal conditions dioctanoyl-*sn*-glycero 3-sulfate is hydrolyzed with a rate of 6000 μmol min^{-1} (mg of protein)$^{-1}$. Although PLA is rather stereoselective in the hydrolysis of anionic diacylglycero sulfates (the *sn*-1 isomer is degraded more than 100 times slower than the *sn*-3 antipode), the high protein concentrations required in direct binding studies and the rather long periods of time involved in these experiments might lead to detectable levels of hydrolysis of the *sn*-1 compounds. In our initial direct binding studies, the enantiomeric dioctanoyl-*sn*-glycero sulfates (I) were used because of the superior



I



II

substrate properties of the *sn*-3 isomer. Low concentrations of I precipitated, however, in the presence of Sr^{2+}, preventing the use of the *sn*-3 isomer. With the *sn*-1 compound, being soluble in Ca^{2+}-containing solutions, however, enzymatic hydrolysis could not completely be prevented. Therefore, a minor chemical modification was introduced in I by substituting the acyl ester functions by carbamoyl ester groups (II). The carbamoyl ester function is known to be absolutely resistant toward PLA attack (Gupta & Bali, 1981), and kinetic experiments demonstrated II to be a pure competitive inhibitor of the enzyme (results not shown). An additional advantage of II is its somewhat more polar character, resulting in a higher solubility in Ca^{2+}- and Sr^{2+}-containing buffer solutions.

The interaction of PLA and its zymogen with submicellar concentrations of II was studied by ultraviolet difference spectroscopy, between pH 5 and pH 10. Figure 1 presents as an example the ultraviolet absorbance difference spectra of PLA produced by increasing concentrations of the carbamoylglycero sulfate at pH 8.5 in the presence of Ca^{2+}.

Very similar patterns were obtained in titrations with the zymogen, although somewhat higher detergent concentrations were required to obtain saturation. With both proteins the

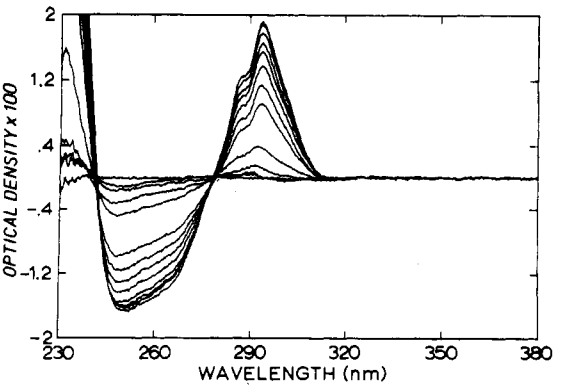


FIGURE 1: Ultraviolet absorbance difference spectra produced by the interaction of porcine pancreatic PLA (22.9 μM) with increasing amounts of *rac*-1,2-(diheptylcarbamoyl)glycero-3-sulfate. Total detergent concentration: 0, 16.9, 33.7, 50.4, 67, 100, 116, 132.3, 148.4, 164.3, 180.2, 195.9, and 211.6 μM. Conditions: 10 mM borate, 20 mM $CaCl_2$, pH 8.5, T = 25 °C.

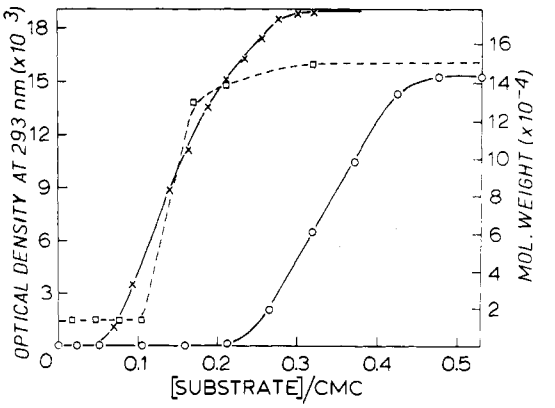


FIGURE 2: Optical density at 293 nm from the ultraviolet absorbance difference spectra at various substrate analogue to cmc ratios [substrate analogue is *rac*-1,2-(diheptylcarbamoyl)glycero 3-sulfate]. cmc of the *rac*-1,2-(diheptylcarbamoyl)glycero sulfate is 0.8 mM in the presence of the divalent cation Ca^{2+}. PLA (22.9 μM), (×) left ordinate; proPLA (22.9 μM), (○) left ordinate. The molecular weight of the premicellar PLA–detergent complexes (□) is given on the right ordinate. Conditions: 10 mM borate, 20 mM $CaCl_2$, pH 8.5, T = 25 °C.

interaction is biphasic. Equilibrium dialysis experiments at low detergent concentrations (results not shown) demonstrated that two substrate molecules are bound with high affinity (K_D = 1.5 μM) to the surface of PLA. From Figure 1 it is clear that this binding hardly perturbs the aromatic chromophores (maximum at 290 nm, minimum at 246 nm). At slightly higher detergent concentrations the difference spectra suddenly change; i.e., the intensity of the difference maxima and minima strongly increase. Moreover, red shifts are observed from 290 to 293 nm (maxima for PLA and the zymogen), from 246 to 250 nm (minimum for PLA), and from 246 to 260 nm (minimum for the zymogen).

A plot of the maximum signal at 293 nm as a function of the detergent/cmc ratio is given in Figure 2 for PLA and its zymogen. Apparently, a much stronger perturbation of aromatic side chains (Trp, Tyr) occurs with both proteins in the second phase.

Molecular weight estimations by equilibrium gel filtration, shown in Figure 2 for PLA only, demonstrate that in the first step of the interaction no protein aggregation occurs. Light scattering and ultracentrifugation measurements confirmed the presence of monomeric PLA and zymogen up to detergent concentrations of 0.05 × cmc and 0.18 × cmc, respectively. Above these well-defined detergent concentrations, a highly

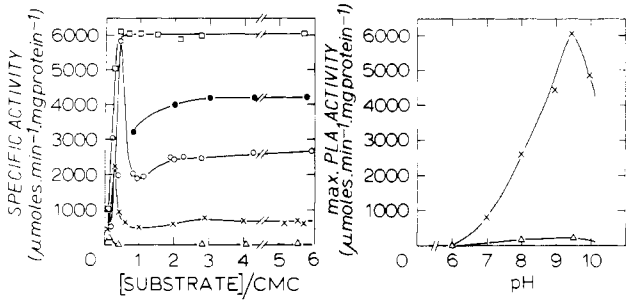


FIGURE 3: (A) Specific activity of PLA at various substrate to cmc ratios (substrate is 1,2-dioctanoyl-*sn*-glycero 3-sulfate) at 6 ≤ pH ≤ 10. cmc of this substrate is 0.3 mM in the presence of divalent cations. pH 6.0 (△), pH 7.0 (×), pH 8.0 (○), pH 9.0 (●), and pH 10 (□). Conditions: 1 mM Pipes (pH 6), 1 mM Hepes (pH 7 and 8), 1 mM borate (pH ≥8.5); 20 mM $CaCl_2$, T = 25 °C. (B) V_{max}–pH profile at 1.8 mM (≈6 × cmc) of 1,2-dioctanoyl-*sn*-glycero 3-sulfate for PLA (×) and proPLA (△) at 25 °C. For conditions see (A).

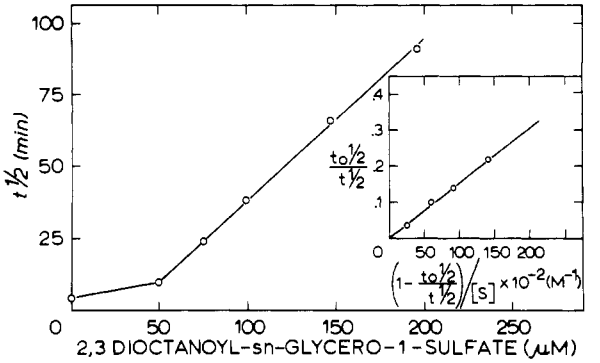


FIGURE 4: Half-times for the inactivation of PLA by phenacyl bromide as a function of 2,3-dioctanoyl-*sn*-glycero 1-sulfate concentration. Conditions: 10 mM Hepes, pH 7.3, 10 mM EDTA, T = 30 °C, [PLA] = 19.3 μM. (Inset) Scrutton and Utter plot for the protection of dioctanoyl-*sn*-glycero sulfate against inactivation. See Materials and Methods.

cooperative interaction process starts in which a number of protein molecules and detergent monomers form complexes with a molecular weight of about 150 000.

In Figure 3A the specific activity of PLA acting on dioctanoylglycero sulfate is recorded as function of the substrate concentration at different pH. It is evident that the enzyme contained in the submicellar high molecular weight aggregates displays very high activity, but only over a narrow substrate concentration range. At pH values ≤8.5, enzymatic activity rapidly decreases at higher detergent concentration, reaches a minimal value around the cmc, and then becomes constant. At pH values ≥9, maximal activity is found below the cmc and remains constant at higher substrate concentrations.

It must be noted that the zymogen, which also forms premicellar high molecular weight complexes, is not activated; enzyme activity is hardly detectable both below and above the cmc of the substrate. Figure 3B gives the "maximal" activity of the enzyme and the zymogen at different pH values, measured at a substrate concentration of 4 × cmc.

In order to obtain information on the affinity of diacylglycero sulfate for the active site of the enzyme, irreversible inhibition of PLA was studied with the haloketone phenacyl bromide in the presence of increasing concentrations of substrate. These studies had to be carried out in the absence of Ca^{2+} to prevent hydrolysis of the substrate. Moreover, Ca^{2+} ions by themselves effectively protect the active center of PLA against attack by haloketones (Volwerk et al., 1973). To be absolutely certain that enzymatic hydrolysis could be precluded, these protection studies were done with the *sn*-1 isomer (D-α) of dioctanoylglycero 3-sulfate.

As shown in Figure 4, with up to 40 μM of the substrate analogue hardly any protection occurs, and only at higher glycero sulfate concentrations were increasing half-times obtained. As mentioned earlier, we know that at this low detergent concentration (40 μM) the enzyme binds two glycero sulfate molecules with high affinity. Apparently, these first detergent molecules bind to loci other than the catalytic center. Only at higher lipid concentrations is substrate available for the active site, resulting in protection. A rough estimate of the dissociation constant of the substrate analogue can be obtained by the analysis of Scrutton & Utter (1965) as shown in the inset of Figure 4.

So far, the substrate properties of the C_3 detergents qualitatively resemble those of the glycol analogues described in the preceding paper. The major difference seems to be that the glycerol derivatives are hydrolyzed much more rapidly than the corresponding glycol analogues. Is this effect primarily caused by the presence of two acyl ester chains or is it related to the splitting of a primary ester group in the glycol detergents and of a secondary ester group in the C_3 compounds?

To answer this question, a more extended series of C_3 substrates, varying in polar head group and acyl ester and/or acylthio ester bonds, including both 1,2-diacyl- and 1,3-diacylglycerol isomers, was investigated. Table II summarizes the kinetic properties of six diheptanoylglycerophosphocholines and of the corresponding sulfates.

For the various *lecithins*, three maximal velocities are given: the appropriate V_{max} of the enzyme at pH 6.0 acting on the substrate at concentrations below the cmc; the maximal velocity of the enzyme at pH 6.0 acting on micellar substrate under saturation conditions; the maximal velocity of the enzyme at pH 9.0 acting on micellar substrate under saturation conditions.

For the glycero *sulfates*, two maximal velocities are recorded: The numbers given at pH 6.0 are apparent values. They represent the highest specific activities that could be experimentally obtained at well-defined *sub*micellar substrate concentrations (given in parentheses). These activities should be considered to be minimal values because of the sharp decline in enzymatic activity at slightly higher detergent concentrations. The velocities measured at pH 9.0 are obtained at substrate concentrations well above the cmc. This allows comparison with the maximal activities obtained with the corresponding lecithins.

Discussion

The results obtained from this kinetic study of pancreatic PLA and its zymogen on diacylglycero lipids confirm and extend the conclusions drawn in the preceding paper, which were obtained with monoacyl*glycol* detergents. Table I shows that at alkaline pH micelles of the diacylglycero sulfates are hydrolyzed 10 times more rapidly than the corresponding lecithin aggregates. 1,2-Dioctanoyl-*sn*-glycero-3-sulfate is the best substrate known today for pancreatic PLA, and under optimal conditions a turnover number of 10^5/min can be reached.

From Figures 2 and 3 it seems clear that with the diacylglycero sulfates maximal activities are found again in premicellar multienzyme complexes of high molecular weight containing a number of detergent monomers. The natural zymogen of pancreatic PLA, prophospholipase, is also able to induce lipid + protein aggregation at substrate concentrations below the cmc; however, this related protein does not show strongly enhanced enzymatic properties in the high molecular weight aggregates. Interfacial binding of the proteins per se

Table II: Maximal Activity [μmol min^{-1} (mg of Protein)$^{-1}$] of Porcine Pancreatic Phospholipase A_2 on Isomeric Diacylglycero Sulfates and Diacylglycerophosphocholines Containing *O*-Acyl and *S*-Acyl Ester Bonds in Positions Y and Z[a]

stereoconfiguration[e]	detergent[b]	Y	Z	maximal activity: monomeric substrate, pH 6.0	maximal activity: micellar substrate, pH 6.0	maximal activity: micellar substrate, pH 9.0	cmc (mM)
sn-3	H_2C—Y—CO—R HC—Z—CO—R H_2C—PN	O S S	O S O	10	100	100	1.50
sn-3	H_2C—Y—CO—R HC—Z—CO—R H_2C—PN	O S S	O S O	4	20	24	0.30
sn-3	H_2C—Y—CO—R HC—Z—CO—R H_2C—PN	O S S	O S O	10	100	92	0.53
	H_2C—Y—CO—R HC—PN H_2C—Z—CO—R	O S S	O S O	0.5	5	5	1.70
rac	H_2C—Y—CO—R HC—PN H_2C—Z—CO—R	O S S	O S O	0.22	0.6	1.1	0.30
	H_2C—Y—CO—R HC—PN H_2C—Z—CO—R	O S S	O S O	0.4	2.9	4.9	0.86[c]

stereoconfiguration[e]	detergent	Y	Z	premicellar aggregate, pH 6.0[d]	micellar substrate, pH 9.0	cmc (mM)
sn-3	H_2C—Y—CO—R HC—Z—CO—R H_2C—O—SO_3^-	O S S	O S O	60 (0.7)	900	0.90
sn-3	H_2C—Y—CO—R HC—Z—CO—R H_2C—O—SO_3^-	O S S	O S O	8.5 (0.05)	180	0.16
sn-3	H_2C—Y—CO—R HC—O—SO_3^- H_2C—Z—CO—R	O S S	O S O	56 (0.17)	800	0.33
	H_2C—Y—CO—R HC—O—SO_3^- H_2C—Z—CO—R	O S S	O S O	3.4 (0.6)	50	0.70
rac	H_2C—Y—CO—R HC—O—SO_3^- H_2C—Z—CO—R	O S S	O S O	0.5 (0.06)	9	0.16
	H_2C—Y—CO—R HC—O—SO_3^- H_2C—Z—CO—R	O S S	O S O	1.2 (0.16)	28.5	0.33

[a] R = constant = C_6H_{13}. 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. All values were determined with the titrimetric assay. [b] PN stands for phosphocholine. [c] Solubility problems of this isomer required the presence of 4% ethanol in the aqueous buffer. [d] The submicellar substrate concentrations (mM) at which maximal activity is reached is shown in parentheses. [e] For all substrates marked with the stereoconfiguration *sn*-3, the maximal velocities are derived from those measured on racemic mixtures by multiplication with a factor of 2. See text and Discussion.

is not sufficient for activation of the enzyme, suggesting that a structural rearrangement upon binding to the interface is essential and possible only with the active PLA.

From Table II, the following general conclusions can be drawn: the 1,3-diacylglycerol detergents are hydrolyzed much more slowly than the 1,2-diacyl-*sn*-glycero-3-type analogues; O → S substitution in the cleavable ester bond leads in the diacylglycerol lipids to considerably lower enzymatic activity. The maximal activity values given in Table II were all obtained with the titrimetric assay method. Occasionally, the much more sensitive spectroscopic assay could be used simultaneously, and good agreement was found with both techniques for lecithins and glycero sulfates containing two thio ester bonds. The spectrophotometric method provided additional evidence for the purity of some of the "mixed ester" substrates containing one *O*-acyl and one *S*-acyl ester bond. Hardly any SH release was observed in the PLA-catalyzed hydrolysis of 1-*S*-acyl-2-*O*-acylglycerol derivatives, which proves the absence of the isomeric 1-*O*-acyl-2-*S*-acyl compound. In the racemic 1,3-diacylglycerol lipids, containing one thio ester and one oxy ester linkage, the enzyme acts on the thio ester bond in one of the enantiomers, whereas in the antipode the *O*-acyl ester is cleaved (Slotboom et al., 1976). The titrimetric assay determined the sum of *O*-acyl ester + *S*-acyl ester cleavage, and the observed hydrolysis rate should be equal to the mean value of the diacyl ester and dithioacyl ester compounds (OO + SS)/2. As was expected, the spectroscopic assay yielded values equal to about half of the hydrolysis rate of the dithioacyl ester analogue (SS/2).

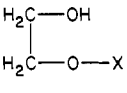
The lower solubility of the diacylglycerol detergents as compared to the monoacylglycol analogues limits, however, the use of the spectrophotometric method. It should also be noted that the specific activities given in Table II for the *sn*-3 isomers have not been measured directly but were derived from those experimentally obtained on the *racemic* mixtures by multiplication by a factor of 2. The correctness of this derivation in the case of pancreatic PLA acting on micellar substrates has been demonstrated previously (Bonsen et al., 1972) and was confirmed in the present study for the hydrolysis of zwitterionic and anionic detergents at substrate concentrations below the cmc. The fact that *sn*-3 substrates and the enantiomeric, nondegradable *sn*-1 isomers bind with identical affinity to the active center of pancreatic PLA and form lipid–water interfaces above the cmc that are physicochemically indistinguishable greatly simplifies the task of the organic chemist interested in the kinetics of lipolysis by this enzyme.

Table II shows that the glycerol sulfates are much better substrates for the enzyme than the corresponding lecithins. At pH 9.0, the anionic sulfates are hydrolyzed 8–10 times faster than the zwitterionic phosphocholine-containing derivatives. The same difference was found for the glycol lipids and confirms the well-known preference of pancreatic PLA for negatively charged substrates. Also at pH 6 and below the cmc of the substrates, the sulfates seem to be hydrolyzed more efficiently than the lecithins.

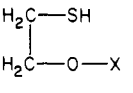
Striking is the preference of the enzyme for 1,2-diacyl-*sn*-glycero-3-type lipids as compared to the isomeric 1,3-diacylglycerol analogues. Both for the sulfates and for the lecithins, the 1,3-diacyl-glycero-2-type derivatives are hydrolyzed with a rate of only 4–5% of that of the corresponding *sn*-3 isomers. As previously demonstrated (Bonsen et al., 1972), this difference is not caused by "K_m" effects, since the 1,2 and 1,3 isomers in micellar and monomeric form bind with the same affinity to the enzyme. Apparently, the primary ester bonds in 1,3-diacylglycero lipids are more resistant toward PLA attack than the secondary ester group in the 1,2-diacyl compounds. The weak substrate properties of glycol detergents

(see foregoing paper) have also primarily been ascribed to the presence of a primary ester group. A possible explanation for the slow hydrolysis of primary ester bonds could be a change in the rate-limiting step of the reaction, i.e., if formation of a tetrahedral intermediate would proceed much more slowly with primary ester bonds, then lower k_{cat} values can be expected.[4] There is one major problem, however, with this explanation. In the case of *O*-acyl derivatives, the C_2 detergents are hydrolyzed at pH 9.0 about 500 times more slowly than the corresponding 1,2-diacyl-*sn*-glycero-3-type analogues. This is found for the sulfates and the phosphocholine-containing lipids. A similar comparison for the *S*-acyl derivatives, however, reveals that the C_2 detergents are degraded only 20 times more slowly than the corresponding *sn*-3-glycero analogues. This tremendous difference is caused by the fact that O → S substitution in the C_2 compounds considerably *improves* substrate properties (factor of 5), whereas the same substitution in the C_3 detergents has the opposite effect and results in a 5 times *lower* maximal velocity. From the values obtained with the 1,2-diacyl detergents (both lecithins and glycero sulfates), it is clear that the drop in enzymatic activity is caused by the single O → S substitution at the glycerol C_2 carbon atom. Single O → S substitution on the glycerol C_1 carbon hardly has any effect on the hydrolysis rate. This latter observation strongly indicates that the lower cmc of the *S*-acyl-containing detergents as compared with the *O*-acyl compounds is not responsible for the increase or decrease in enzymatic activity upon O → S substitution.

How can we explain this peculiar difference between glycol and glycerol detergents? For the C_2 compounds, the increase in V_{max} upon O → S substitution has been ascribed to the better leaving properties of the SH-containing product, assuming that decomposition of the tetrahedral intermediate is the rate-limiting step in catalysis. For venom and pancreatic PLA's, a covalent acyl–enzyme intermediate could never be trapped (Wells, 1971, 1972). This is in agreement with the high-resolution X-ray structures of these enzymes, which show the absence of serine in the active site (Dijkstra et al., 1981a,b, 1983; Keith et al., 1981). We may assume, however, that PLA-catalyzed hydrolysis proceeds via a tetrahedral intermediate (Verheij et al., 1980). Wells (1972), for example, convincingly demonstrated that the rate-limiting step in venom PLA catalyzed hydrolysis of lecithin is the decomposition of the tetrahedral intermediate with the fatty acid release first followed by the lysolecithin. Most probably, the pancreatic enzyme follows the same mechanism, and this would explain the better substrate properties of the thio ester containing glycol lipids. There seems to be no reason to assume a different behavior for the C_3 detergents. The fact that with the 1,2-diacylglycerol and the 1,3 derivatives O → S substitution results in severalfold lower activities argues against a change in rate-limiting steps for substrates containing a cleavable secondary of primary ester group. Taking all results obtained with the pancreatic PLA together, we must conclude that most probably this enzyme follows the same reaction mechanism independently of the nature of the polar head group, the presence of *O*-acyl or *S*-acyl ester groups, and whether the substrate is of the C_2 or C_3 type. This implies that the origin of the difference between the C_2 and C_3 detergents must be sought in the binding *affinities* of the products to the catalytic site of the enzyme. The fact that all substrates compiled in Table II possess the same acyl chain length proves that slow *fatty acid* release from the enzyme surface can be precluded. The other breakdown product, however, is different for C_2 and C_3 substrates. With the glycol detergents a very polar compound remains (structures III and IV; X is phosphocholine

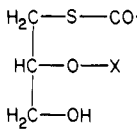


III

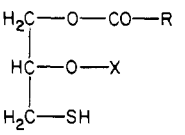


IV

or sulfate); thus, both for the sulfates and for the phosphocholine-containing detergents it can be expected that these hydrolysis products possess no affinity to the catalytic site and are immediately released from the enzyme. After hydrolysis of the C_3 substrates, however, the resulting lyso compounds still contain one apolar acyl chain, and it cannot be precluded that the combination of one *S*-acyl chain + free OH (structure V; X is phosphocholine or sulfate) possesses a different affinity



V



VI

to the enzyme as compared to the isomer with one *O*-acyl chain + free SH group (structure VI; X = phosphocholine or sulfate). In fact, from our synthetic work (solubility behavior in different solvents, R_f values in TLC), we have strong indications that the free SH in medium-chain (partial) glycerides has a very apolar character and nearly behaves as a –S–C(O)–R group! Therefore, we predict that lyso-C_3 lipids containing a free SH group (e.g., VI) will possess a higher affinity to the enzyme, resulting in a lower breakdown rate. Work is in progress with isomeric lysolecithins to furnish direct proof.

Registry No. (*R*)-1,2-Dihexanoyl-3-PN, 34506-67-7; (*R*)-1,2-diheptanoyl-3-PN, 39036-04-9; (*R*)-1,2-dioctanoyl-3-PN, 19191-91-4; (*R*)-1,2-dinonanoyl-3-PN, 27869-45-0; (*S*)-1,2-diheptanoyl-3-PN, 89019-63-6; (*S*)-1-thioheptanoyl-2-heptanoyl-3-PN, 99165-68-1; 1,3-diheptanoyl-2-PN, 35387-75-8; 1,3-dithioheptanoyl-2-PN, 99165-69-2; (±)-1-thioheptanoyl-3-heptanoyl-2-PN, 99212-67-6; (*R*)-$CH_3(CH_2)_4CO_2CH_2CH[O_2C(CH_2)_4CH_3]CH_2OSO_3H$, 99165-65-8; (*R*)-$CH_3(CH_2)_5CO_2CH_2CH[O_2C(CH_2)_5CH_3]CH_2OSO_3H$, 99165-66-9; (*R*)-$CH_3(CH_2)_6CO_2CH_2CH[O_2C(CH_2)_6CH_3]$-$CH_2OSO_3H$, 99165-67-0; (±)-$PhCH_2OCH_2CH(OH)CH_2OH$, 13071-59-5; $HOCH(CH_2SH)_2$, 584-04-3; (±)-$HSCH_2CH(SH)$-CH_2OH, 81600-56-8; (±)-$HOCH_2CH(OH)CH_2SH$, 53023-42-0; $CH_3(CH_2)_5C(O)Cl$, 2528-61-2; (*S*)-$CH_3(CH_2)_5C(O)SCH_2CH[S$-$C(O)(CH_2)_5CH_3]CH_2OSO_3H$, 99165-70-5; (*S*)-$CH_3(CH_2)_5C(O)S$-$CH_2CH[O_2C(CH_2)_6H]CH_2OSO_3H$, 99165-71-6; $HO_3SOCH[CH_2$-$OC(O)(CH_2)_6H]_2$, 99165-72-7; $HO_3SOCH[CH_2SC(O)(CH_2)_6H]_2$, 99165-73-8; (±)-$CH_3(CH_2)_5C(O)SCH_2CH(OSO_3H)CH_2O_2C$-$(CH_2)_5CH_3$, 99165-74-9; phenacyl bromide, 70-11-1; phospholipase A_2, 9001-84-7; prophospholipase A_2, 37350-21-3.

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[4] A possible reason for the slow hydrolysis of the primary ester bonds might be the presence of the adjacent phosphate (or sulfate) group. These polar moieties possess a high electron density and a negative charge that could counteract the charge polarization of the carbonyl group of the cleavable ester linkage (Brockerhoff, 1968).

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Slow Refolding Kinetics in Yeast Iso-2 Cytochrome *c*†

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ABSTRACT: In refolding of iso-2 cytochrome *c* from *Saccharomyces cerevisiae*, there are two slow folding reactions, τ_{1a} and τ_{1b}. The slower of the slow reactions, τ_{1a} = 100–200 s, is observed only by absorbance changes, while τ_{1b} (10–20-fold faster) is detected by fluorescence changes. The temperature dependence of the rates of these reactions has been measured: for kinetic experiments ending below the folding–unfolding transition zone (pH 7.2, 0.3 M guanidine hydrochloride, 5–30 °C), the activation enthalpies are $\Delta H^{\ddagger}$ = 27 kcal/mol for τ_{1a} and 21 kcal/mol for τ_{1b}. Double-jump (unfolding, then refolding) experiments demonstrate that the two sets of species responsible for the slow folding reactions are generated slowly but at different rates under unfolding conditions (3 M guanidine hydrochloride, pH 7.2, 20 °C). Finally, as a test for changes in the population of the slow refolding species under different unfolding conditions, the amplitudes for slow refolding have been measured as a function of the initial unfolding conditions with the final refolding conditions held constant. Over the range accessible to measurement in the absence of interference from other reactions, the amplitudes for fluorescence-detected (α_{1b}) and absorbance-detected (α_{1a}) slow folding are independent of guanidine hydrochloride concentration and pH in the initial conditions. Although a full description requires a more complex explanation, many of the properties of the slow folding species are those expected for proline imide bond isomerization.

The proposal that the slow kinetic phases associated with protein folding–unfolding reactions are due entirely to isomerization of proline imide bonds (Brandts et al., 1975) is subject to a variety of experimental tests. Interpreted strictly, the isomerization hypothesis requires that the properties of slow folding reactions in proteins be identical with those of proline imide bond isomerizations. Generally, this has proved not to be the case. Even the slow folding reactions of ribonuclease A, the protein chosen for the initial tests of the hypothesis, are as often at variance as in agreement with the properties expected for proline isomerization (Nall et al., 1978; Schmid & Baldwin, 1979; Cook et al., 1979; Kim & Baldwin, 1980; Schmid, 1981, 1983; Schmid & Blaschek, 1981; Schmid et al., 1984; Lin & Bandts, 1983a–c, 1984; Nall, 1985).

The kinetic behavior of the folding reactions of horse cytochrome *c* presents a more pronounced discrepancy. In this case, it has been known for some time that chemical modification of Met-80 (a heme ligand in the native protein) specifically increases the rate of slow refolding while leaving the fast reactions unaltered (Ikai, 1971) and that removal of the iron atom eliminates slow kinetic phases entirely (Henkins & Turner, 1979). Recently, an investigation of the pH and extrinsic ligand dependence of the refolding of horse cytochrome *c* has been reported (Brems & Stellwagen, 1983). They find that an intermediate kinetic phase in refolding of

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