de Vendittis, E., Palumbo, G., Parlato, G., & Bocchino, V. (1981) *Anal. Biochem. 115*, 278–286.
Donnér, J., Spink, C. H., Borgström, B., & Sjöholm, I. (1976) *Biochemistry 15*, 5413–5417.
Donovan, J. W. (1969) in *Physical Principles and Techniques of Protein Chemistry* (Leach, S. J., Ed.) Part A, pp 101–170, Academic Press, New York.
Egmond, M. R., Slotboom, A. J., de Haas, G. H., Dijkstra, K., & Kaptein, R. (1980) *Biochim. Biophys. Acta 623*, 461–466.
Hayashi, K. (1975) *Anal. Biochem. 67*, 503–506.
Heinrikson, R. L., Krueger, E. T., & Keim, P. A. (1977) *J. Biol. Chem. 252*, 4913–4921.
Hendrickson, H. S., Trygstad, W. M., Loftness, T. L., & Sailer, S. L. (1981) *Arch. Biochem. Biophys. 212*, 508–514.
Hille, J. D. R., Donné-Op den Kelder, G. M., Sauve, P., de Haas, G. H., & Egmond, M. R. (1981) *Biochemistry 20*, 4068–4073.
Hille, J. D. R., Egmond, M. R., Dijkman, R., van Oort, M. G., Sauve, P., & de Haas, G. H. (1983) *Biochemistry* (following paper in this issue).
Jansen, E. H. J. M., Meijer, H., de Haas, G. H., & Kaptein, R. (1978) *J. Biol. Chem. 253*, 6346–6347.
Jirgensons, B., & de Haas, G. H. (1977) *Biochim. Biophys. Acta 494*, 285–292.
Kaptein, R. (1978) in *Nuclear Magnetic Resonance Spectroscopy in Molecular Biology* (Pullman, S., Ed.) pp 211–229, Reidel, Dordecht, The Netherlands.
Nieuwenhuizen, W., Kunze, H., & de Haas, G. H. (1974) *Methods Enzymol. 32B*, 147–154.
Nikolov, A., Martynov, G., Ekserova, D., & Kaishev, V. (1981) *Colloid J. USSR (Engl. Transl.) 42*, 559–565.
Panicheva, L. P., & Markina, Z. N. (1982) *Colloid J. USSR (Engl. Transl.) 43*, 538–543.
Pieterson, W. A., Vidal, J. C., Volwerk, J. J., & de Haas, G. H. (1974) *Biochemistry 13*, 1455–1460.
Roberts, M. F., Deems, R. A., & Dennis, E. A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1950–1954.
Roberts, M. F., Adamich, M., Robson, R. J., & Dennis, E. A. (1979) *Biochemistry 18*, 3301–3308.
Scrutton, M. C., & Utter, M. F. (1965) *J. Biol. Chem. 240*, 3714–3729.
Smith, C. M., & Wells, M. A. (1981) *Biochim. Biophys. Acta 663*, 687–694.
Teshima, K., Ikeda, K., Hamaguchi, K., & Hayashi, K. (1981) *J. Biochem. (Tokyo) 89*, 1163–1174.
van Dam-Mieras, M. C. E., Slotboom, A. J., Pieterson, W. A., & de Haas, G. H. (1975) *Biochemistry 14*, 5387–5393.
van Deenen, L. L. M., & Haas, G. H. (1963) *Biochim. Biophys. Acta 70*, 538–553.
Verger, R., & de Haas, G. H. (1973) *Chem. Phys. Lipids 10*, 127–136.
Verger, R., & de Haas, G. H. (1976) *Annu. Rev. Biophys. Bioeng. 5*, 77–117.
Verger, R., Mieras, M. C. E., & de Haas, G. H. (1973) *J. Biol. Chem. 248*, 4023–4034.
Verheij, H. M., Slotboom, A. J., & de Haas, G. H. (1981) *Rev. Physiol., Biochem. Pharmacol. 91*, 91–203.
Volwerk, J. J., & de Haas, G. H. (1982) in *Lipid-Protein Interactions* (Jost, P. C., & Griffith, O. H., Ed.) Vol. I, pp 69–149, Wiley, New York.
Volwerk, J. J., Pieterson, W. A., & de Haas, G. H. (1974) *Biochemistry 13*, 1446–1454.

Unusual Kinetic Behavior of Porcine Pancreatic (Pro)phospholipase A_2 on Negatively Charged Substrates at Submicellar Concentrations†

Jan D. R. Hille, Maarten R. Egmond, Ruud Dijkman, Maarten G. van Oort, Paul Sauve,‡ and Gerard H. de Haas*

ABSTRACT: The negatively charged detergents *S*-*n*-alkanoylthioglycol sulfates (C_8, C_9, and C_{10}) are substrates for porcine pancreatic phospholipase A_2 and its zymogen. At pH 6.0 and detergent concentrations up to 0.08 × critical micelle concentration (cmc), the activities of active enzyme and zymogen are similar and very low. From 0.08 × cmc to 0.12 × cmc a tremendous increase in activity is observed for phospholipase A_2, but not for the zymogen. Concomitant with this increase in activity there is a sharp rise in molecular weight of the substrate–enzyme complex, from 15 000 to 95 000, and in detergent to protein molar ratio of 1:1 to about 7:1. This indicates both substrate and enzyme aggregation. Most probably a lipid–water interface is formed inside the aggregated protein particle by which the enzyme is activated. Although the zymogen also forms high molecular weight complexes with similar molar ratios, no activation is observed probably because of distortion of its lipid binding domain.

In the preceding paper (Hille et al., 1983) the interaction of porcine pancreatic phospholipase A_2 and its zymogen with sodium *n*-alkyl sulfates was studied at submicellar detergent concentrations. The interaction was shown to involve two steps. First, the protein binds with high affinity about two detergent molecules. This binding causes an increased hydrophobicity of the protein surface resulting in a second step in which high molecular weight complexes are formed, consisting in the case of the active enzyme on average of 6 enzyme molecules and about 40 detergent monomers and for the zymogen on average of 4 protein molecules and about 30 detergent monomers.

Although this aggregation takes place at detergent con-

† From the Laboratory of Biochemistry, State University of Utrecht Transitorium 3, "De Uithof", Padualaan 8, NL-3584 CH Utrecht, The Netherlands. *Received October 21, 1982.* These investigations were partly carried out under the auspices of The Netherlands Foundation of Chemical Research (SON), with financial aid from The Netherlands Organization for the Advancement of Pure Research (ZWO).

‡ Present address: Centre de Biologie Moléculaire C.N.R.S. 31, Chemin J. Aiguier, 13274 Marseille, France.

0006-2960/83/0422-5353$01.50/0

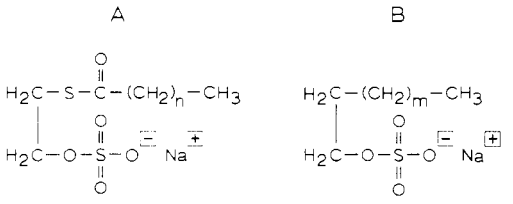

FIGURE 1: Structural formulas of sodium *S-n*-alkanoylthioglycol sulfates (A) with n = 6, 7, or 8 and of sodium *n*-alkyl sulfates (B) with m = 7 or 9.

centrations far below the cmc,[1] the spectral changes strongly resemble those observed previously in binding studies of the enzyme with *micellar* zwitterionic substrates (van Dam-Mieras et al., 1975). This latter interaction is known to strongly activate PA_2. Therefore, it was of interest to investigate whether complex formation at submicellar lipid concentrations also results in enzyme activation. For this purpose a detergent is required whose structure strongly resembles that of *n*-alkyl sulfate but contains a (thio) ester function cleavable by phospholipase A_2. Keeping in mind the minimal substrate requirements of the enzyme (van Deenen & de Haas, 1963), a series of *S-n*-alkanoylthioglycol sulfates (Figure 1A) has been synthesized. The thio ester group was preferred over the normal oxygen ester function, because of the sensitive continuous spectrophotometric assay of liberated SH groups (Aarsman et al., 1976; Volwerk et al., 1979). The *S-n*-alkanoylthioglycol sulfates turned out to be good substrates for the enzyme and were used to study the kinetic behavior of phospholipase A_2 at submicellar detergent concentrations.

Materials and Methods

Porcine prophospholipase A_2 and phospholipase A_2 were purified in accordance with established procedures (Nieuwenhuizen et al., 1974). Protein concentrations were calculated from the absorbance at 280 nm by using $E^{1\%}_{1cm}$ values of 12.5 (phospholipase A_2) and 12.3 (prophospholipase A_2). DTNB was obtained from Aldrich, and 4-PDS was a Fluka product.

Synthesis of Substrates. Synthesis of S-n-Decanoylthioglycol Sulfate (Sodium Salt). A 39-g sample of 2-mercaptoethanol (0.5 mol) and 39 g of pyridine (0.5 mol) were dissolved in 300 mL of dry chloroform. After cooling to 0 °C, 0.45 mol of decanoyl chloride dissolved in 100 mL of chloroform was added dropwise under stirring, keeping the temperature below 3 °C. Subsequently the reaction mixture was extracted successively with 100-mL portions of H_2O, 1 M HCl, H_2O, 5% $NaHCO_3$, and H_2O. After drying over anhydrous $MgSO_4$, the chloroform was evaporated in vacuo, and the residual colorless oil was dissolved in 400 mL of 96% ethanol and stored overnight at −20 °C. The crystalline fraction, consisting of the didecanoyl derivative of 2-mercaptoethanol,[2] was collected by filtration, and the filtrate was evaporated in vacuo. The residue was dissolved in 250 mL of pentane and stored at −20 °C. The crystalline *S-n*-decanoylthioglycol (mp 22.0 °C) was isolated in a yield of 35%,[3] and DTNB analysis showed that less than 0.1% of free SH groups were present.

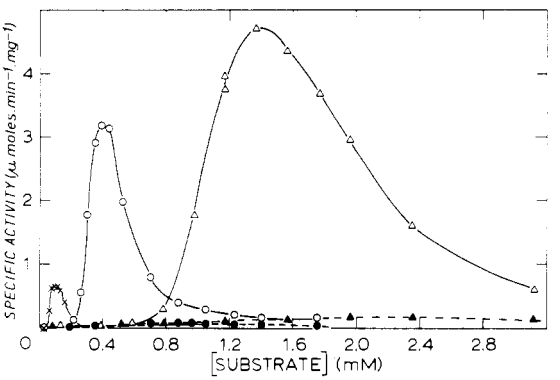

FIGURE 2: Specific activity of porcine pancreatic phospholipase A_2 (open symbols) and its zymogen (closed symbols) as a function of the substrate concentration at 25 °C. Protein concentration was 0.8 μM. Substrates used were *S-n*-octanoylthioglycol sulfate (△), *S-n*-nonanoylthioglycol sulfate (○), and *S-n*-decanoylthioglycol sulfate (×).

A 17-g sample of *S-n*-decanoylthioglycol (78 mmol) was dissolved in 200 mL of dry chloroform. After addition of 16 g (200 mmol) of pyridine, the mixture was cooled to 0 °C; 6.7 mL (11.6 g) of freshly distilled chlorosulfonic acid (0.1 mol) was slowly added under vigorous stirring keeping the temperature at 0 °C. Excess chlorosulfonic acid was destroyed with ice–water. After washing with water the chloroform phase was dried over $MgSO_4$. The chloroform was evaporated in vacuo, and the residue was dissolved in water, keeping the pH at 7.5 by adding aqueous NaOH. Pyridine and H_2O were subsequently removed by lyophilization: yield 100%. The sodium salt was purified by recrystallization from 96% ethanol or 2-propanol. Anal. Calcd for $C_{12}H_{23}O_5S_2Na$: C, 43.11; H, 6.89; O, 23.95; S, 19.16. Found: C, 42.72; H, 6.93; O, 24.28; S, 19.04. The syntheses of the *S-n*-octanoyl- (OTGS) and *S-n*-nonanoyl- (NTGS) derivatives proceeded analogously.

Critical micelle concentration values were obtained in buffer solutions by measuring the surface tension as a function of the substrate concentration by using the Wilhelmy plate technique (Davies & Rideal, 1961) and/or by means of the soluble fluorescent probe ANS as described by de Vendittis et al. (1981). The buffers used were the following: 0.1 N sodium acetate, pH 6.0, and 25 mM $CaCl_2$ (or 25 mM $SrCl_2$) in fluorescence, gel chromatography, and equilibrium dialysis studies; 10 mM sodium acetate (pH 5.0), 10 mM Pipes (pH 6.0), 10 mM Hepes (pH 7 and 8), and 25 mM $CaCl_2$ (or 25 mM $SrCl_2$) in kinetic analyses and in UV absorbance difference spectroscopy studies.

Kinetic Analysis. The hydrolysis of these thio ester substrates catalyzed by the enzyme or its zymogen was followed in a spectrophotometric assay described by Volwerk et al. (1979). DTNB above pH 7.5 and 4-PDS below pH 7.5 were used as chromophores. Activities were calculated by using an ϵ_{412} of 13 600 for DTNB and an ϵ_{324} of 19 800 ($M^{-1}\cdot cm^{-1}$) for 4-DPS.

Sedimentation Velocity. All sedimentation experiments were performed on a Beckman Model E ultracentrifuge equipped with interference and schlieren optics. A double-sector synthetic boundary cell was used for sedimentation velocity experiments at rotations between 48 000 and 36 000

[1] Abbreviations: PA_2, phospholipase A_2; IRS, interface recognition site; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); 4-PDS, di-4-pyridyl disulfide; cmc, critical micelle concentration; ANS, 8-anilino-1-naphthalenesulfonic acid, magnesium salt; EDTA, ethylenediaminetetraacetic acid; OTGS, *S-n*-octanoylthioglycol sulfate, sodium salt; NTGS, *S-n*-nonanoylthioglycol sulfate, sodium salt; DTGS, *S-n*-decanoylthioglycol sulfate, sodium salt; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPTLC, high-performance thin-layer chromatography.

[2] Notwithstanding a molar excess of 2-mercaptoethanol in all preparations about 50% of the acyl chloride was recovered as the diacyl derivative.

[3] HPTLC of the filtrate showed the presence of about 15% of the more soluble *O-n*-decanoylthioglycol.

Table I: Critical Micelle Concentrations of the Thio Ester Substrates in One Buffer System at 25 °C and pH 6.0 and the Apparent Hydrolysis Constants for the Activated Enzyme–Substrate Complex under These Conditions

substrate	cmc (mM)[a,b]	substrate/cmc ratio at the highest sp act.	apparent hydrolysis constants[b] K_D[c] (μM)	maximal sp act. (μmol·min^{-1}·mg^{-1})
OTGS	11.2	0.12	0.6	6.7
NTGS	3.5	0.12	0.8	6.0
DTGS	1.0	0.13	1.0	1.5

[a] In 10 mM Pipes and 25 mM $CaCl_2$. [b] The SD for all values is about 5%. [c] This dissociation constant reflects the amount of enzyme needed to aggregate half the number of substrate monomers.

rpm. All experiments were carried out at 25 °C in 10 mM sodium acetate and 10 mM $BaCl_2$.

UV absorbance difference and fluorescence spectroscopy, equilibrium dialysis, and equilibrium gel filtration were carried out as described in the preceding paper (Hille et al., 1983).

Results

Figure 2 shows the results of the hydrolysis of three thio ester substrates (Figure 1A) at pH 6.0 and 25 °C by porcine pancreatic PA_2 and its zymogen. The critical micelle concentrations of these substrates under the conditions used are given in Table I.

The overall picture is the same for all three substrates. At very low substrate concentrations below 0.1 × cmc, the zymogen and PA_2 show similar and very weak hydrolysis rates. For each substrate at about 0.1 × cmc the specific activity of phospholipase A_2 sharply increases, reaches a maximum at 0.12–0.13 × cmc, and falls off at higher detergent concentration. This behavior is in contrast with that of the zymogen which shows hardly any increase in specific activity at higher substrate concentrations.

The experiments described in Figure 2 were carried out at a constant protein concentration of 0.8 μM both for the zymogen and for PA_2. At different protein concentrations similar bell-shaped curves were obtained, all with maximal specific activities at a constant substrate concentration of 0.12–0.13 × cmc. However, no linear proportionality was observed between enzymatic activity and PA_2 concentration. As demonstrated in Figure 3 for NTGS the specific activity of the enzyme, measured at the optimal substrate concentration of 395 μM (see Figure 2), increases hyperbolically with enzyme concentration. A similar behavior was observed for the other two substrates. The specific activity of the zymogen, however, turned out to be independent of protein concentration. Nonlinear proportionality between enzyme activity and enzyme concentration is indicative of enzyme aggregation.

From the saturation curves, as shown in Figure 3, "maximum specific activities" and "dissociation constants" were calculated by unweighted least-squares analysis of Hanes' plots. These values are listed in Table I. It should be pointed out already that these "constants" are apparent (see Discussion). From Table I and Figure 2 it is striking that the maximal enzyme activities are rather similar for the C_8 and C_9 thio esters and considerably higher than for the C_{10} homologue. Kinetic studies on monomolecular surface films of a series of lecithins (Pattus et al., 1979) also showed optimal enzyme activity for the di-C_9-lecithin. Most probably it is this

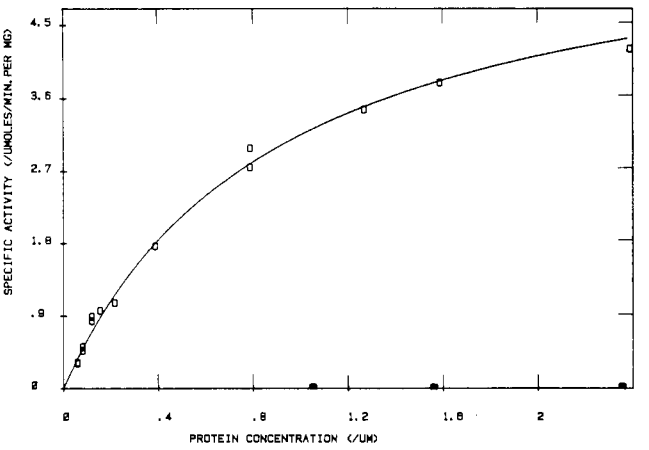

FIGURE 3: Specific activity as a function of the phospholipase A_2 concentration (□) and the zymogen (■) at pH 6.0. The substrate used was *S-n*-nonanoylthioglycol sulfate at a concentration of 395 μM. The drawn line is a fit of a nonlinear regression analysis to eq 8 as given in a previous paper (Hille et al., 1981). This results in a K_D = 0.8 μM and an apparent maximal specific activity of 6.0 μmol·min^{-1}·mg^{-1}.

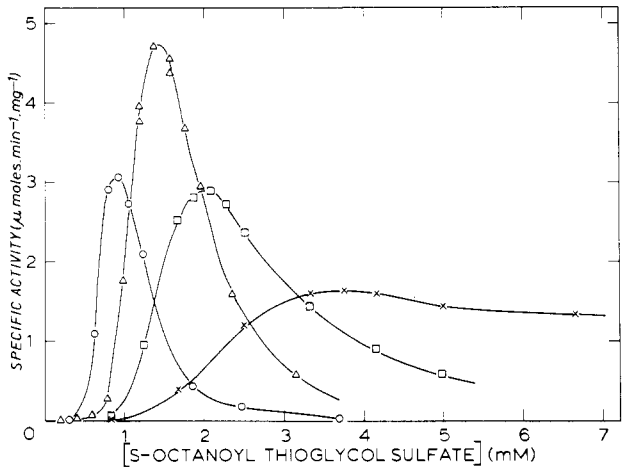

FIGURE 4: pH dependence of the specific activity of phospholipase A_2 (0.8 μM) on *S-n*-octanoylthioglycol sulfate. The symbols used are for pH 5.0 (○), pH 6.0 (△), pH 7.0 (□), and pH 8.0 (×).

chain length which optimally fits the catalytic center of the enzyme.

Figure 4 shows how the specific activity of phospholipase A_2 as a function of OTGS concentration varies with pH. It is clear that higher pH values lower the tendency of the enzyme to aggregate into the productive enzyme–substrate complexes and higher detergent concentrations are required to get optimal specific activities. Most probably this behavior is related to the slightly acidic isoelectric point of the enzyme (pH 6.3). The pH dependence of the specific activity of the aggregated enzyme shows optimal activity at pH 6.0. This pH optimum was also found with neutral zwitterionic lecithins (de Haas et al., 1971). In order to get more insight into the aggregation process, direct binding studies were performed either in the presence of the competitive inhibitor Sr^{2+} or Ba^{2+} (Pieterson et al., 1974) or in the absence of divalent cations by adding 2 mM EDTA to the buffer solutions. The results for the active enzyme obtained from equilibrium gel filtration, sedimentation velocity, and equilibrium dialysis are summarized in Figure 5.

The various techniques were applied to substrates having different acyl chain lengths. However, when the substrate concentrations were divided by their respective cmc values under the conditions used, one general picture could be ob-

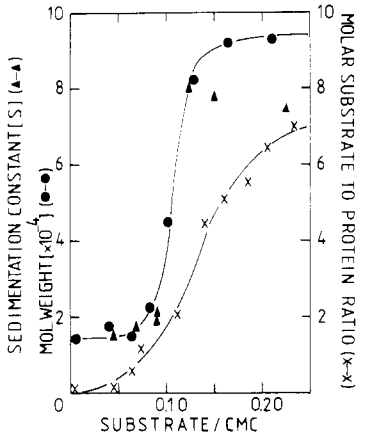

FIGURE 5: Molecular weight (●), sedimentation constant (▲) (in svedberg units), and substrate to protein molar ratio (×) for the complex formed between porcine pancreatic phospholipase A_2 and the various thio ester substrates at pH 6.0 and 25 °C.

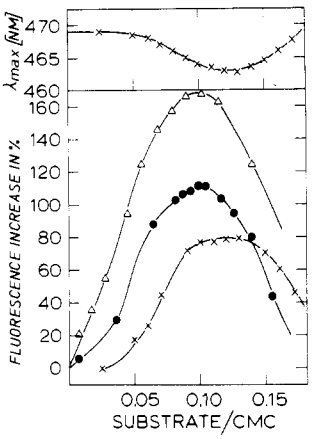

FIGURE 6: ANS fluorescence at various substrate to cmc ratios in the presence of 7.5 μM phospholipase A_2 at pH 6.0. The substrates used were OTGS (×), NTGS (●), and DTGS (▲). The shift in emission maximum for the interaction of phospholipase A_2 and OTGS is shown in the upper panel.

tained (cf. Table I). Compared to the *n*-alkyl sulfates described in the preceding paper (Hille et al., 1983), the thio ester substrates behaved similarly in their interaction with PA_2 and its zymogen. The binding of the thio ester substrate occurs in a two-step process. First, about two detergent molecules bind to PA_2 with high affinity. The molecular weight of the complex hardly changes as determined by gel filtration and ultrasedimentation. At detergent concentrations around 0.08 × cmc the molecular weight of the complex strongly increases to a value of about 95 000, coinciding with an increase in the substrate to protein molar ratio to about 7. This points to aggregates consisting in average of about 6 enzyme molecules and 40 detergent monomers. The zymogen forms comparable complexes in a two-step process with the thio ester substrates at a molar ratio of 6–7 detergent monomers per protein molecule. These complexes possess a somewhat lower molecular weight of about 75 000, indicating aggregates formed of 4 protein molecules and about 30 detergent monomers.

Finally, it has to be pointed out that the two-step binding process of thio ester substrates to PA_2 and its zymogen can be followed also by UV absorbance difference spectroscopy and by fluorescence spectroscopy (data not shown). For both proteins the interaction gives rise to a *decrease* in fluorescence quantum yield in both binding steps in contrast to the increased quantum yield observed upon binding of *n*-alkyl sulfates. Most probably the sulfur atom of the thio ester bond quenches the Trp fluorescence when it approaches the indole ring of Trp.

The kinetic data of Figure 2 together with the direct binding results shown in Figure 5 suggest that the "superactivation" of the enzyme parallels the formation of the high molecular weight complexes. This is true, however, only for the ascending parts of the curves. While the direct binding curves tend to saturate at substrate concentrations of about 0.2 × cmc, the specific activity profiles show a bell-shaped curve with a pronounced maximum at about 0.12–0.13 × cmc.

To get some more insight in the structure of the high molecular weight complexes, the water-soluble fluorescent probe ANS was added to the system. This anionic dye is almost nonfluorescent in water but becomes highly fluorescent in micellar structures or when bound to macromolecules. The fluorescence quantum yield as a function of the thio ester concentration is shown in Figure 6 (lower panel). For all three substrates bell-shaped profiles were obtained having maximum fluorescence and a maximal blue shift of the emission maximum as shown in Figure 6 (upper panel) at thio ester concentrations of 0.10–0.12 × cmc, indicating the incorporation of ANS. The exact substrate concentration where maximum fluorescence increase occurs is slightly dependent on the ANS concentration. However, after extrapolation to zero dye concentration, the fluorescence curves coincide with the kinetic profiles shown in Figure 2.

The combined results of Figures 2, 5, and 6 indicate that at thio ester concentrations of about 0.08 × cmc enzyme *and* detergent aggregation occurs, resulting in high molecular weight aggregates. At about 0.12 × cmc these aggregates exhibit maximal enzyme activation *and* ANS fluorescence quantum yield. When the well-known activation of pancreatic PA_2's by organized lipid–water interfaces (Pieterson et al., 1974) and the fluorescence behavior of ANS are taken into account, the most direct explanation for the observed results implies the formation of a "pseudomicellar" detergent core, induced and stabilized by aggregated enzyme molecules.

Discussion

The direct binding studies of pancreatic PA_2 with *n*-alkyl sulfates (Hille et al., 1983) and with thio ester containing alkyl sulfates (present study) show that this enzyme possesses a few so-called "hot" sites where about two anionic detergent monomers (D) bind with very high affinity.

$$\sum_{i=0}^{1}(ED_i + D \rightleftarrows ED_{i+1}) \qquad (1)$$

The spectroscopic results (Trp perturbation) leave little doubt that this binding takes place at the exposed hydrophobic surface patch of the enzyme, previously called interface recognition site (Pieterson et al., 1974).

Although porcine PA_2 by itself, even in rather concentrated aqueous solutions, hardly displays any tendency to aggregate, occupation of these hot sites by apolar alkyl chains and the supposed neutralization of both positive charges in the IRS must be responsible for the initiation of a second process: enzyme aggregation and clustering of detergent molecules. Although both the pancreatic PA_2 and its zymogen demonstrate this behavior, a strongly enhanced catalytic activity that accompanies the aggregation process is observed only for the active enzyme. Notwithstanding our complete ignorance of the possible existence of several intermediates it appears that for the various thio ester substrates complexes are formed, exhibiting high kinetic activity at a well-defined detergent concentration. These aggregates consist of about 6 enzyme molecules and on average 40 detergent monomers.[4]

$$\sum_{i=0}^{1}\sum_{j=3}^{5}[6(ED_{i+1} + jD) \rightleftarrows E_6D_6^*{}_{(i+1+j)}] \quad (2)$$

For the various thio ester substrates maximal enzyme activities at pH 6.0 are found at substrate concentrations of about 0.12 × cmc (Figure 2 and Table I) independent of the acyl chain length.[5] This indicates that the pseudomicellar detergent core within the aggregate must owe its stability at least partly to forces similar to those controlling micelle stability and cmc. Finally, in the aggregated enzyme–substrate complex effective hydrolysis occurs with an observed rate ν

$$\sum_{i=0}^{1}\sum_{j=3}^{5} E_6{}^*D_{6(i+1+j)} \xrightarrow{\nu} E + \text{products} \quad (3)$$

in which E stands for all nonactive enzyme complexes. The catalytic "constants" K_D and maximal specific activity compiled in Table I were derived from saturation curves as shown in Figure 3. These curves describe the aggregation process of eq 2, leading to the superactivated particle $E_6{}^*D_{6(i+1+j)}$. The unexpected hyperbolic relationship between specific activity and total enzyme concentration, found for all three thio ester substates, can be explained if the concentration of superactivated enzyme particles is small as compared to the total enzyme concentration. It is interesting to note that for the various thio esters at a constant substrate concentration of 0.12 × cmc, the amount of enzyme needed to aggregate half of the number of detergent monomers is roughly constant (K_D = 0.8 ± 0.2 μM).

It should be realized, however, that although increase of total PA_2 concentration leads to more superactivated particles and therefore to higher "specific activity" values, the extrapolated "maximal specific activities" of Table I probably still do not represent the real turnover numbers of the enzyme in the substrate–protein aggregates. From eq 3 it follows that ν equals $V_{max}(k_{cat})$ only when the active site of the enzyme molecules are fully saturated by substrate monomers. Although the aggregated enzyme molecules face a pseudomicellar core of substrate monomers (see below), the detergent concentration is constant and cannot be varied. The experimental finding (data not shown) that thio ester concentrations of 0.12 × cmc hardly protect the active center of the enzyme against active-site-directed irreversible inhibitors (cf. Volwerk et al., 1974) strongly indicates a weak affinity of the catalytic site for these single-chain substrates. This result is in agreement with previously determined dissociation constants of single-chain substrate analogues for pancreatic phospholipase A_2. Both *n*-alkylphosphocholines (van Dam-Mieras et al., 1975) and *n*-alkyl sulfates [see Hille et al. (1983)] bind to the active center of the enzyme with dissociation constants in the *millimolar* range. Therefore, it is doubtful whether in the aggregated enzyme–detergent complexes the active sites of the protein molecules will be saturated by substrate monomers.

The large increase in PA_2 activity over a relatively narrow concentration of substrate (cf. Figure 2) is evidence of a cooperative process and is an indication that a binding-induced conformational change has occurred. Independent of acyl chain length, maximal enzyme activity is found at pH 6.0 at a substrate concentration of 0.12 × cmc, and the kinetically most active particle seems to consist of 6 enzyme molecules surrounding a pseudomicellar core of about 30 substrate monomers. This interpretation is supported by the ANS fluorescence results of Figure 6. For all three thio ester substrates increasing detergent concentrations first give rise to higher fluorescence quantum yields and a blue shift of the emission maximum, indicating the incorporation of ANS in a hydrophobic structure.[6] Maximal fluorescence is observed at about 0.10 × cmc.

One question remains, why enzyme activity and fluorescence quantum yield decrease at higher substrate concentrations. A possible reason could be an increasing disintegration of the kinetically active particle and loss of the pseudomicellar substrate core. The red shift of the maximal fluorescence wavelength indicates indeed that at higher substrate concentrations ANS is no longer incorporated in a hydrophobic environment, and disruption of the organized substrate structure would explain the rapid decrease of catalytic activity. From Figure 5 it is clear, however, that the average molecular weight of the complexes remains high and roughly constant at about 95 000. Therefore, complete disruption of the aggregated particles must be excluded.

A second explanation for the bell-shaped kinetic profile of Figure 2 might be inhibition by excess substrate. As the physiological substrates of phospholipase A_2 are *di*acylglycerophosphatides, one might imagine that with *single*-chain detergents such as *S*-*n*-alkanoylthioglycol sulfates more than one molecule can bind to the active center, causing inhibition. Because of the close correlation observed between the kinetic and fluorescence profiles, one must then assume that the second substrate molecule binding to the active center displaces at the same time an already bound ANS molecule. However, both kinetic experiments and direct binding studies have demonstrated that ANS has only a very weak affinity for the pancreatic enzyme and does not bind to the active center. Although excess substrate inhibition cannot be absolutely excluded, a reliable kinetic investigation is prevented by the complexity of the present system consisting of aggregated protein and substrate molecules.

Another question arises: is superactivation due to enzyme-induced substrate aggregation and/or to a substrate-induced phospholipase aggregation? This problem appears to be a semantic question, but we have to take into account one important experimental finding: both the zymogen and PA_2 form high molecular weight protein–detergent complexes, but only PA_2 becomes superactivated. At this stage it is impossible to preclude that the superactivation of pancreatic PA_2 in the aggregated complexes is primarily caused by protein-protein interaction, a mechanism discussed for some venom phospholipases A_2 (Smith & Wells, 1981; Dennis et al., 1981; van Eijk et al., 1983). However, a different explanation for the observed results, which would be in line with our previous interpretations (Pieterson et al., 1974; Volwerk & de Haas, 1982), involved the recognition by the (aggregated) PA_2 molecules of a lipid–water interface formed by the clustered thio ester detergent monomers. The specific interaction between the IRS and certain well-defined interfaces is supposed to induce a conformational change in the enzyme with a concomitant optimization of the active site (Pieterson et al., 1974). Indirect evidence is accumulating (Dijkstra et al., 1981; Verheij et al., 1980; van Scharrenburg et al., 1981) that even subtle changes in amino acid residues of the IRS can strongly influence the catalytic machinery. Although the

[4] With the asterisk in E^* we want to indicate the possibility of a conformational change in the enzyme E induced by the aggregation process.

[5] Fluorescence stopped-flow spectroscopy showed half-times of 300 ms for the second step in the interaction (Hille et al., 1983). The formation of the active hexamer is fast enough, and the activity profile shown will not be an artifact of complex formation.

[6] Although ANS binds very weakly to PA_2, addition of only a few substrate molecules is already sufficient to replace ANS by the detergent (data not shown).

zymogen also forms high molecular weight aggregates with the anionic detergents, its distorted IRS possibly prevents the correct interaction with the pseudomicellar detergent core, and the catalytic site is not improved.

It has to be pointed out that superactivation by anionic detergents has been reported for other enzymes as well. D'yakov et al. (1980) demonstrated that low concentrations of sodium dodecyl sulfate activate pancreatic lipase, but they did not investigate possible aggregation of the enzyme.

Smith (1982) showed that (self)association of myelin basic protein is increased at low molar ratios (1 to 2) of dodecyl sulfate, deoxycholate, Triton X-100, and lysophosphatidylcholine.

Calame et al. (1975) found that single-chain sterol ester hydrolase from rat pancreas is enzymatically inactive. Addition of bile salts, however, induced aggregation of the enzyme molecules, and the catalytically active species was demonstrated to be a hexamer.

Recently van Eijk et al. (1983) showed that PA_2 from *Naja melanoleuca* forms tetramers in the presence of submicellar concentrations of zwitterionic substrate analogues. Moreover, high enzymatic activities were found with short-chain lecithins at substrate concentrations below the cmc. Our approach differs from those studies in that the activating detergents used for pancreatic PA_2 are at the same time substrates for the enzyme. The key result of the present study is that the superactivated PA_2 is present in a particle containing about 6 enzyme molecules and 40 substrate monomers.

We are currently investigating the kinetic behavior of porcine pancreatic PA_2 on 1,2-diacyl-(C_7,C_8)-*sn*-glycero-3-sulfates. The first experiments show that comparable complexes are formed as described in this paper and that these negatively charged lipids are much better substrates for this enzyme than the corresponding zwitterionic phosphatidylcholines: 2–3 times higher specific activities are found. Also the localization of the hot sites is in current progress.

Registry No. DTGS, 87116-14-1; OTGS, 87116-15-2; NTGS, 87116-16-3; PA_2, 9001-84-7; prophospholipase A_2, 37350-21-3; *S*-*n*-decanoylthioglycol, 87116-17-4; 2-mercaptoethanol, 60-24-2; decanoyl chloride, 112-13-0; didecanoyl derivative of 2-mercaptoethanol, 7362-41-6.

References

Aarsman, A. J., van Deenen, L. L. M., & van den Bosch, H. (1976) *Bioorg. Chem. 5*, 241–253.

Calame, K. B., Gallo, L., Cheriathundam, E., Vahouny, G. V., & Treadwell, C. R. (1975) *Arch. Biochem. Biophys. 168*, 57–65.

Davies, J. T., & Rideal, E. K. (1961) in *Interfacial Phenomena*, pp 46–47, Academic Press, New York.

de Haas, G. H., Bonsen, P. P. M., Pieterson, W. A., & van Deenen, L. L. M. (1971) *Biochim. Biophys. Acta 239*, 252–266.

Dennis, E. A., Darke, P. L., Deems, R. A., Kensil, C. R., & Plückthun, A. (1981) *Mol. Cell. Biochem. 36*, 37–43.

de Vendittis, E., Palumbo, O., Parlato, G., & Bocchino, V. (1981) *Anal. Biochem. 115*, 278–286.

Dijkstra, B. W., Drenth, J., & Kalk, K. H. (1981) *Nature (London) 289*, 604–606.

D'yakov, V. L., Mishin, A. A., & Antonov, V. K. (1980) *Sov. J. Bioorg. Chem. (Engl. Transl.) 6*, 157–159.

Hille, J. D. R., Donné-Op den Kelder, G. M., Sauve, P., de Haas, G. H., & Egmond, M. R. (1981) *Biochemistry 20*, 4068–4073.

Hille, J. D. R., Egmond, M. R., Dijkman, R., van Oort, M. H., Jirgensons, B., & de Haas, G. H. (1983) *Biochemistry* (preceding paper in this issue).

Nieuwenhuizen, W., Kunze, H., & de Haas, G. H. (1974) *Methods Enzymol. 32B*, 147–154.

Pattus, F., Slotboom, A. J., & de Haas, G. H. (1979) *Biochemistry 18*, 2691–2697.

Pieterson, W. A., Vidal, J. C., Volwerk, J. J., & de Haas, G. H. (1974) *Biochemistry 13*, 1455–1469.

Smith, C. M., & Wells, M. A. (1981) *Biochim. Biophys. Acta 663*, 687–694.

Smith, R. (1982) *Biochemistry 21*, 2697–2701.

van Dam-Mieras, M. C. E., Slotboom, A. J., Pieterson, W. A., & de Haas, G. H. (1975) *Biochemistry 14*, 5387–5393.

van Deenen, L. L. M., & de Haas, G. H. (1963) *Biochim. Biophys. Acta 70*, 538–553.

van Eijk, J. H., Verheij, H. M., Dijkman, R., & de Haas, G. H. (1983) *Eur. J. Biochem. 132*, 183–188.

van Scharrenburg, G. J. M., Puijk, W. C., Egmond, M. R., de Haas, G. H., & Slotboom, A. J. (1981) *Biochemistry 20*, 1584–1591.

Verheij, H. M., Volwerk, J. J., Jansen, E. H. J. M., Puijk, W. C., Dijkstra, B. W., Drenth, J., & de Haas, G. H. (1980) *Biochemistry 19*, 743–750.

Volwerk, J. J., & de Haas, G. H. (1982) in *Lipid–Protein Interactions* (Jost, P. C., & Griffith, O. H., Eds.) Vol. I, pp 69–149, Wiley, New York.

Volwerk, J. J., Pieterson, W. A., & de Haas, G. H. (1974) *Biochemistry 13*, 1446–1454.

Volwerk, J. J., Dedieu, A. G. R., Verheij, H. M., Dijkman, R., & de Haas, G. H. (1979) *Recl. Trav. Chim. Pays-Bas 98*, 214–220.