Aggregation of Porcine Pancreatic Phospholipase A_2 and Its Zymogen Induced by Submicellar Concentrations of Negatively Charged Detergents†

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

ABSTRACT: The interaction of two sodium *n*-alkyl sulfates (C_{10} and C_{12}) with porcine pancreatic phospholipase A_2 and its zymogen was studied by various spectroscopic techniques, equilibrium gel filtration, calorimetry, and photochemically induced dynamic nuclear polarization ^1H NMR. At very low concentrations of *n*-alkyl sulfate [up to 0.07 × critical micelle concentration (cmc)] the enzyme (M_r 14 000) is able to build up a complex with the detergent molecules having a molecular weight of about 90 000. This complex consists of 6 enzyme molecules and about 40 *n*-alkyl sulfate monomers. The formation of the detergent–protein aggregate occurs in a two-step process: First, two detergent molecules strongly bind to a hydrophobic surface region of the protein, previously called interface recognition site [Pieterson, W. A., Vidal, J. C., Volwerk, J. J., & de Haas, G. H. (1974) *Biochemistry 13*, 1455–1460]. Subsequently, at higher detergent concentrations suddenly 6 enzyme molecules aggregate, probably including about 30 additional detergent monomers. Although the zymogen of the pancreatic phospholipase A_2 seems to form comparable high molecular weight aggregates with these detergents, there are spectroscopic differences, and higher detergent concentrations are required. Moreover, as will be shown in the following paper [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)], only the phospholipase A_2 becomes superactivated in these complexes.

Phospholipases A_2 (EC 3.1.1.4) catalyze the specific hydrolysis of fatty acid ester bonds at the 2-position of 3-*sn*-phosphoglycerides (de Haas et al., 1968) and require Ca^{2+} ions as essential cofactor. They belong to a special class of esterases, the lipolytic enzymes. Although these enzymes are able to catalyze the hydrolysis of molecularly dispersed substrates, they become superactivated in the presence of certain organized lipid–water interfaces (Brockerhoff & Jensen, 1974; Verger & de Haas, 1976). Up to now no generally accepted explanation has been given for the extremely effective heterogeneous catalysis. The pancreatic phospholipases A_2 are known to possess a cluster of hydrophobic amino acid side chains at the surface of the protein. This apolar patch called interface recognition site (IRS)[1] is topographically distinct from the catalytic center and anchors the enzyme to the lipid–water interface. It has been proposed that this interaction gives rise to a conformational change in the protein with a concomitant optimization of the active site (Pieterson et al., 1974; Verger et al., 1973).

The naturally occurring zymogens of the pancreatic phospholipases A_2 hydrolyze molecularly dispersed substrates as efficiently as the active enzyme itself. However, the unique spatial configuration of the IRS is perturbed, and the zymogen is not able to interact with zwitterionic lipid–water interfaces.

In order to identify the amino acid side chains involved in the recognition of, and interaction with, lipid–water interfaces, numerous direct binding studies have been carried out with micellar solutions of nondegradable substrate analogues (cf. Volwerk & de Haas, 1982). Mostly neutral phosphocholine-containing lipids have been applied to allow the presence of Ca^{2+} ions which do not perturb the lipid organization.

Moreover, the wish to include spectroscopic techniques dictated optically clear solutions which can be obtained only with short-chain diacyllecithins or long-chain monoacylphosphocholine derivatives. Although these studies have yielded a rather detailed picture of the lipid binding site of the enzyme (Verheij et al., 1981), there are several reasons to include negatively charged lipids as well.

In vivo the enzyme acts in the intestine on bile salt stabilized phospholipid micelles. These conjugated bile salts confer a strong negative charge on the phospholipid dispersion even at pH 6.0. Moreover, also in the absence of bile salts the pancreatic enzymes are known to attack preferentially anionic phospholipids (van Deenen & de Haas, 1963; de Haas et al., 1968; Bonsen et al., 1972). This preference is most clearly demonstrated by kinetic studies on monomolecular surface films (Verger & de Haas, 1973; Hendrickson et al., 1981). Taking into account that diacyl-*sn*-glycero-3-sulfates are much better substrates for the pancreatic PA_2's than the corresponding lecithins (unpublished observation), we have investigated the binding properties of *n*-alkyl sulfates to PA_2 and its zymogen. It will be shown that high-affinity binding of only two detergent molecules to the IRS of the enzyme increases its surface hydrophobicity to such an extent that enzyme aggregation occurs. The enzyme molecules aggregate probably by inclusion of a "pseudomicellar" detergent core,

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

‡ Deceased Oct 8, 1982. Last affiliated with the Department of Biochemistry, The University of Texas, System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, TX 77030.

[1] Abbreviations: IRS, interface recognition site; PA_2, phospholipase A_2; SDS, sodium *n*-decyl sulfate; SDDS, sodium *n*-dodecyl sulfate; cmc, critical micelle concentration; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CIDNP, chemically induced dynamic nuclear polarization; TSP, [2,2,3,3-$^2H_4$](trimethylsilyl)propionate; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; ANS, 8-anilino-1-naphthalenesulfonic acid.

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although the total detergent concentration is far below the critical micelle concentration.

Materials and Methods

Porcine pancreatic prophospholipase A_2 was isolated and converted into active enzyme as described by Nieuwenhuizen et al. (1974). Protein concentrations were calculated from the absorbance at 280 nm with an $E^{1\%}_{1cm}$ = 12.3 and 12.5, for precursor and active enzyme, respectively.

Sodium *n*-decyl sulfate and sodium *n*-dodecyl sulfate were prepared by sulfonation of the respective pure alcohols with chlorosulfonic acid and purified by crystallization from ethanol. The ^{35}S-labeled decyl sulfate was prepared in the same way by sulfonation with chloro[^{35}S]sulfonic acid (from Amersham Int. Ltd., U.K.). The *n*-alkyl sulfate concentrations were determined with Methylene Blue according to Hayashi (1975) or from radioactivity measurements by using a Searle Isocap 300 scintillation counter. Counting vials contained 50–200 μL of the solution and 3 mL of Instagel (Packard). Critical micelle concentrations were determined by using the Wilhelmy plate method (Davies & Rideal, 1961) and/or means of the soluble fluorescent probe ANS as described by de Vendittis et al. (1981). ANS was obtained from Eastman-Kodak Co.

The buffers used at pH 6.0 were either 0.1 M sodium acetate and 25 mM calcium chloride (in gel filtration, equilibrium dialysis, calorimetry, and fluorescence spectroscopy) or 10 mM Pipes and 25 mM calcium chloride (in UV absorbance difference spectroscopy). At pH 8.0, 10 mM Hepes and 25 mM calcium chloride were used.

Fluorescence spectra were measured at 25 °C with a Perkin-Elmer MPF-3 spectrofluorometer by using 1-cm cells. The excitation wavelengths were 370 (for ANS) and 295 nm [for (pro)phospholipase A_2]. The emission spectra were recorded from 460 to 510 nm and from 320 to 360 nm, respectively.

Fluorescence stopped-flow spectroscopy was done on a Durrum stopped-flow fluorometer at pH 6.0 and 25 °C. When equal volumes of 0.1 mL containing pancreatic PA_2 (10.7 μM) and various concentrations of sodium *n*-decyl sulfate were mixed, the formation of detergent–protein aggregates was followed. The excitation wavelength was 295 nm. A cutoff filter for transmission of wavelengths above 330 nm was used. Transients were recorded and stored by using a MINC computer linked to the fluorometer.

UV absorbance 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; 12-bit paralleled data transfer from the MIDAN to the computer was accomplished, by using either the "PLOT" mode (slow transfer 1 min) or "CRT" mode (fast transfer 30 ms) of the MIDAN. Transferred spectra were stored on floppy disks (1.5 KB per spectrum). Measurements were done, and binding parameters were obtained as previously described by de Araujo et al. (1979) and Hille et al. (1981).

Equilibrium Gel Filtration. A column (0.9 × 30 cm) was packed with Sephadex G-100 superfine, equilibrated at 25 °C in 0.1 M sodium acetate at pH 6.0, 25 mM $CaCl_2$, and various concentrations of ^{35}S-labeled *n*-alkyl sulfates. When the column was used in an upward flow direction, a constant amount of enzyme, dissolved in the equlibrium buffer, was passed through the column. From the elution volume the molecular weight could be estimated after calibration of the column with a Boehringer protein Combithek size II unit. From the overlap in enzyme activity or OD_{280} profile and the detergent peak the detergent to protein molar ratio of the complex could be calculated according to the procedure described by Pieterson et al. (1974).

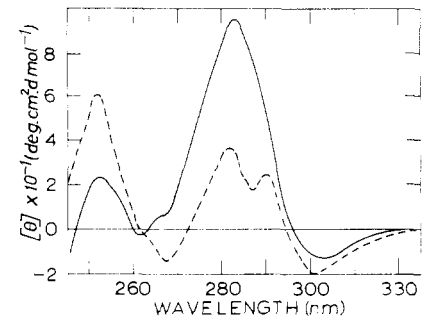

FIGURE 1: Circular dichroism spectra of porcine pancreatic phospholipase A_2 (63 μM) (—) and of a 1:1 molar mixture of PA_2 and sodium *n*-dodecyl sulfate (---). The buffer used was 0.05 M sodium acetate, pH 5.8, and 0.1 M NaCl at 25 °C.

Equilibrium Dialysis. Another way to determine the detergent to protein molar ratio is equilibrium dialysis. In a Dianorm Equilibrium Dialyzer equipped with 10 two-compartment twin cells, 0.2 mL of a (pro)enzyme solution (20 μM) was added to one side of the membrane (Diachema, M_r cutoff 5000) and 0.2 mL of various concentrations of ^{35}S-labeled *n*-decyl sulfate to the other side. The membranes were wetted by pretreatment with the equilibrium dialysis buffer. After 2–3 h of dialysis when equlibrium had been reached, the content of each compartment was recovered. After determination of the detergent concentrations the lipid to protein ratios could be calculated.

Circular dichroism measurements were carried out as described by Jirgensons & de Haas (1977).

Microcalorimetry. Microcalorimetric measurements were performed as described by de Araujo et al. (1979) by using an LKB type 2107 batch microcalorimeter, equipped with gold cells and thermostated at 15, 25, or 35 °C. A Keithley 150B microvoltmeter coupled with an LKB 2066 recorder was used to amplify and record the output from the calorimeter. All measurements were done in the 10- or 3-μV range of the voltmeter. The calibration was performed electrically and checked by measuring the heat of dilution of sucrose. The calorimetric cells were charged by using Gilson P 5000 pipets which were gravimetrically calibrated to deliver 2.0 and 4.0 mL of the solution used. In all the experiments 2.0 mL of a (pro)enzyme solution (about 0.8 mg/mL) in buffer was mixed with 4.0 mL of a *n*-decyl sulfate solution at various concentrations in the same buffer. The reference cell was charged in the same way, except that no protein was added. Separate dilution experiments were made with the reactants.

Photo-CIDNP ^1H NMR. The ^1H NMR (360 MHz) photo-CIDNP spectra obtained with a Bruker HX-360 spectrometer at the SON facility in Groningen, The Netherlands, were recorded as described before (Kaptein, 1978; Jansen et al., 1978; Egmond et al., 1980) by using 3-(carboxymethyl)-lumiflavin as the dye. Chemical shifts were calculated relative to TSP by using 3.764 ppm for the chemical shift difference between TSP and dioxane. The latter was added as internal standard.

Enzyme inactivation by phenacyl bromide was done in 0.1 M cacodylate–HCl buffer, pH 6.4, containing 2 mM EDTA at 32 °C according to Volwerk et al. (1974).

Results

The first indication that porcine pancreatic phospholipase A_2 possesses a high affinity for monomeric *n*-alkyl sulfate was obtained by circular dichroic spectroscopy. As shown in Figure 1, the addition of an equimolar amount of *n*-dodecyl sulfate to PA_2 considerably perturbs the tertiary structure of the

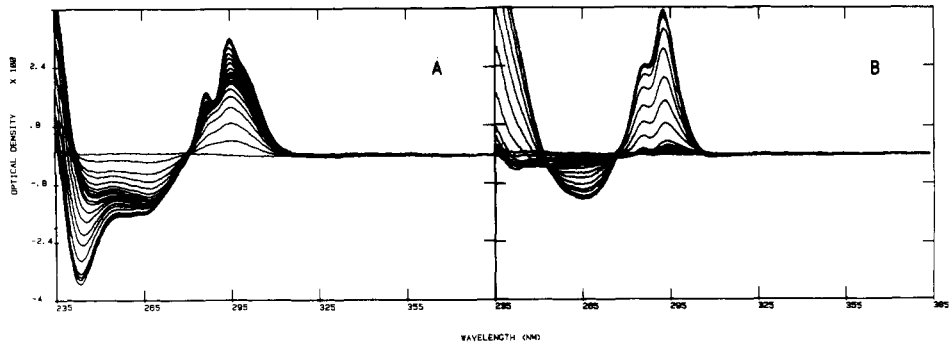

FIGURE 2: Ultraviolet absorbance difference spectra produced by the interaction of porcine pancreatic phospholipase A_2 (35.3 μM) (A) and prophospholipase A_2 (32.7 μM) (B) with increasing amounts of sodium *n*-decyl sulfate at pH 6.0 and 25 °C. In panel A the detergent concentrations are in between 0 and 450 μM and in panel B in between 0 and 850 μM.

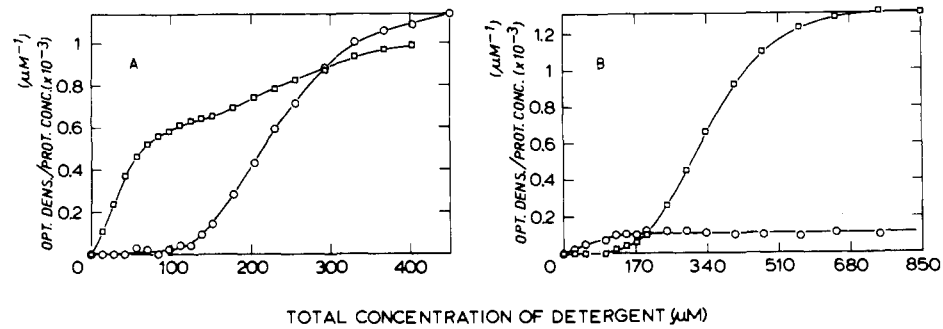

FIGURE 3: Optical density (divided by the protein concentration) at different wavelengths from the UV absorbance difference spectra as a function of the total detergent concentration. (A) The interaction with phospholipase A_2 at 295 nm (□) and at 242 nm (○). (B) The interaction with prophospholipase A_2 at 293 nm (□) and at 253 nm (○).

enzyme. The CD bands in the 270–295-nm region are diminished, while the band near 250 nm is strongly enhanced. Appearance of band fine structure near 290 nm and the shift and enhancement of the negative trough from 310 to 300 nm are noteworthy and indicate that tryptophan and tyrosine side chains are involved in the binding of the detergent. This is confirmed by UV absorbance difference spectroscopy. Figure 2A shows the UV absorbance difference spectra obtained by adding increasing amounts of sodium *n*-decyl sulfate (SDS) to a solution of porcine pancreatic PA_2. The cmc of SDS under these conditions is 9.6 mM. At the lower concentrations of SDS up to 150 μM, i.e., far below the cmc, the difference spectra are characterized by absorbance peaks at 288 and 295 nm, a trough around 265 nm, and isosbestic points at 243 and 281 nm. Such difference spectra strongly resemble those obtained upon interaction of PA_2 with micelles of the zwitterionic substrate analogues, the *n*-alkyl phosphocholines (van Dam-Mieras et al., 1975; Hille et al., 1981).

The micellar difference spectra were shown to have their origin in a hydrophobic perturbation of the single Trp residue and of one or more tyrosine side chains belonging to the interface recognition site. Therefore, it seems that the enzyme possesses at least one high-affinity binding site for the negatively charged detergent molecule which is located close to the IRS.

At higher concentrations of SDS, but still far below the cmc, the difference spectra suddenly change. There is a blue shift of the peak at 295 nm, the shoulder at 288 nm becomes more pronounced and shifts to 285 nm, and a deep trough develops at 242 nm. Another shoulder shows up around 305 nm. A plot of the optical densities at 295 and 242 nm as a function of total detergent concentration is shown in Figure 3A. At both wavelengths a change in the pattern occurs at about 130 μM SDS. Apparently there are (at least) two steps in the interaction between PA_2 and SDS.

In order to gain some further insight into the spectroscopic changes produced during the second step of the interaction, the contribution to the difference spectrum of the first binding step had to be eliminated. From Figure 3A it is clear that the initial process is nearly terminated at about 130 μM SDS, and therefore, the second process can be followed by correcting the optical densities at all wavelengths for the contribution to the difference spectrum caused by 130 μM SDS. From the results (data not shown) it was clear that the second step is characterized by a pure tryptophan perturbation very similar to that obtained by the transfer of tryptophan from water to a 20% (v/v) ethylene glycol environment (Donovan, 1969).

When it was taken into account that the zymogen of pancreatic PA_2 does not bind to organized, zwitterionic lipid–water interfaces, probably because of its distorted IRS, it was of considerable interest to study the interaction of this protein with molecularly dispersed anionic detergents. From the UV difference spectra, shown in Figure 2B, it is clear that also the precursor of PA_2 interacts with these negatively charged substrate analogues. The difference signals at low detergent concentrations are very small. At higher concentrations the signal strongly increases while again tryptophan and tyrosines are perturbed. In Figure 3B this two-step interaction is clearly seen: the first step can be followed at 253 nm while the second step is observed at, e.g., 293 nm. There are two isosbestic points at 253 and 276 nm, respectively, while peaks arise at 284 and 293 nm. The microenvironment of the tryptophan residue in the zymogen is less perturbed than in the active enzyme, and there is a stronger perturbation of one or more tyrosine residues. Another difference between zymogen and active enzyme is the amount of detergent needed to obtain maximum signal in the second part of the process (see Table I).

Fluorescence Spectroscopy. By use of the fact that pancreatic (pro)phospholipases A_2 contain only one Trp residue

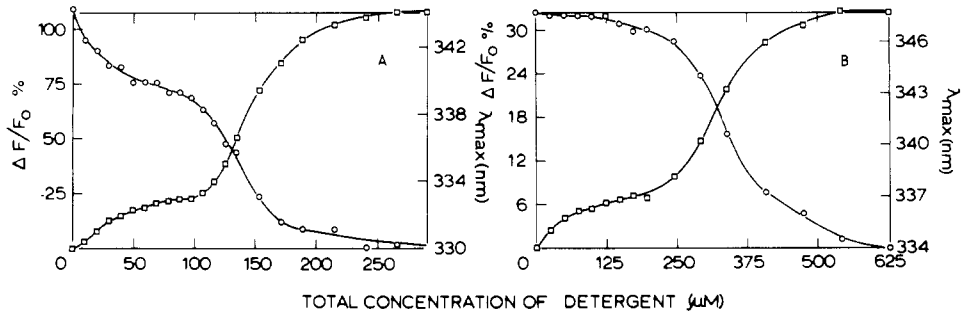

FIGURE 4: Relative fluorescence intensity (□) and the emission maximum (○) of the interaction of porcine pancreatic phospholipase A_2 (A) and prophospholipase A_2 (B) with sodium *n*-decyl sulfate at 25 °C and pH 6.0. The buffer contained 0.1 M sodium acetate and 25 mM $CaCl_2$. Excitation wavelength was 295 nm.

Table I: Influence of Ca^{2+} Ions and pH on the Binding Parameters[a] for the Interaction of Porcine Pancreatic Phospholipase A_2 and Sodium *n*-Decyl Sulfate at 25 °C Studied by UV Difference Spectroscopy and Fluorescence Spectroscopy

		step I			step II
pH	$CaCl_2$	N	K_D (μM)	C (M^{-1} · cm^{-1}) at 293 nm	saturating SDS concn (μM)
(A) Influence of Ca^{2+} Ions and pH on the Binding Parameters Using UV Difference Spectroscopy[b]					
6.0	+	1.9	3.4	692	420
	−	1.9	3.4	738	600
8.0	+	2	115	332	700
	−	2	102	251	900
(B) Influence of pH on the Binding Parameters Using Fluorescence Spectroscopy[c]					
6.0	+	2	13.7		750
8.0	+	2	296		1600

[a] The binding parameters are defined as the following: N, the number of detergent monomers per enzyme molecule in the complex; K_D, the dissociation constant for the detergent–protein complex; C, the molar UV difference extinction coefficient. [b] The buffers used were 10 mM Pipes (pH 6) or Hepes (pH 8) and 25 mM $CaCl_2$ or 2 mM EDTA. [c] The buffers used were 10 mM Pipes (pH 6) or Hepes (pH 8) and 25 mM $CaCl_2$.

in their sequence which is perturbed both by binding of lecithin micelles and by binding of anionic detergent molecules, fluorescence spectroscopy was applied to study the binding of sodium decyl sulfate to these proteins.

The results for porcine PA_2 and its zymogen are shown in parts A and B of Figure 4, respectively. For both proteins the biphasic character of the binding process is evident. The interaction of the active enzyme and SDS at concentrations up to 130 μM gives rise to a small increase of about 20% in quantum yield and a small blue shift of the emission maximum of about 4 nm. At higher detergent concentrations the quantum yield sharply rises and reaches a maximum of about 125%. Concomitantly a further blue shift of the emission maximum of about 10 nm is observed. For the zymogen (Figure 4B) the first step of the binding process hardly perturbs the Trp environment. Above 250 μM SDS the fluorescence quantum yield increases 30%, and again a blue shift of the emission maximum of about 10 nm occurs.

Similar results were obtained in interaction studies with the longer chain homologue sodium *n*-dodecyl sulfate. Again a two-step binding process is observed, and the fluorescence parameters indicate a more apolar microenvironment of Trp[3] upon completion of the interaction.

Equilibrium Gel Filtration and Equilibrium Dialysis. More quantitative information on the interaction between porcine

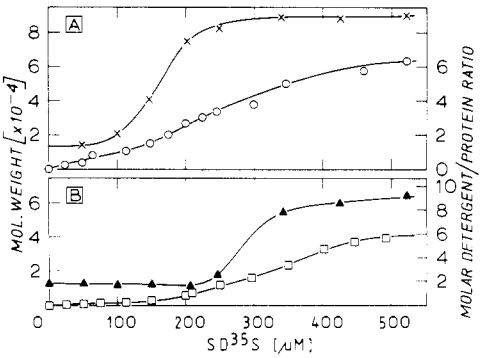

FIGURE 5: Molecular weight of the complex formation between porcine pancreatic phospholipase A_2 (32 μM) (A) (×) or prophospholipase A_2 (30 μM) (B) (▲) and sodium *n*-decyl [^{35}S]sulfate is shown as a function of the total detergent concentration. The detergent to protein molar ratio as a function of free detergent concentration is shown for phospholipase A_2 (35 μM) in panel A (○) and/or prophospholipase A_2 (40.6 μM) in panel B (□).

PA_2 or its zymogen and S(D)DS was obtained from equilibrium gel filtration and equilibrium dialysis with radiolabeled detergents. In Figure 5A the results are shown for SDS binding to the active enzyme. The molecular weight of the detergent–protein complex hardly changes in the first step of the binding process. This is in agreement with the results of equilibrium dialysis showing the presence of only one to two detergent monomers bound per protein molecule. Above 100 μM SDS the molecular weight of the complex suddenly increases simultaneously with the detergent to protein molar ratio. After completion of the second step of the binding process the complex shows a molecular weight of about 90 000 with a detergent/protein molar ratio of 6 to 7.[2]

Rather similar results were obtained in interaction studies of the zymogen with SDS. As shown in Figure 5B, up to 200 μM SDS the molecular weight does not change and in the first step one to two detergent monomers are bound to the protein molecule. At higher detergent concentrations the molecular weight of the complex increases to about 70 000 and the detergent to protein molar ratio rises again to 6. The somewhat lower molecular weight of the zymogen–SDS complex as compared to that found for the active enzyme–detergent aggregate might indicate a different composition (see discussion).

As the molecular weights of the protein–detergent complexes during the first step of the binding process hardly change, protein aggregation can be excluded. This allows a more detailed analysis of the binding parameters between enzyme and detergent by nonlinear regression analysis as described

[2] A molecular weight of about 90 000 was also found in a sedimentation equilibrium experiment for a mixture of 30 μM PA_2 and 200 μM SDDS.

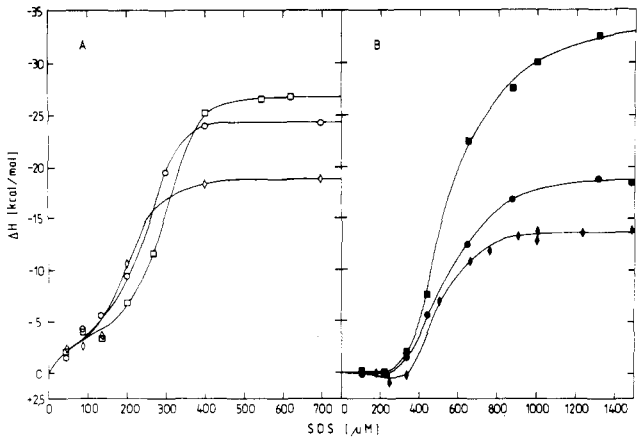

FIGURE 6: Enthalpy changes for porcine pancreatic phospholipase A_2 (A) (open symbols) and prophospholipase A_2 (B) (closed symbols) as a function of the total sodium *n*-decyl sulfate concentration at various temperatures: (□) 35 ± 0.2, (○) 25 ± 0.2, and (◇) for 15 ± 0.2 °C. The enthalpy changes for the zymogen–*n*-decyl sulfate interaction at 15 °C are extra corrected for a small heat of dilution found only for the zymogen at this temperature ($\Delta H_{dil} \sim -3$ kcal/mol).

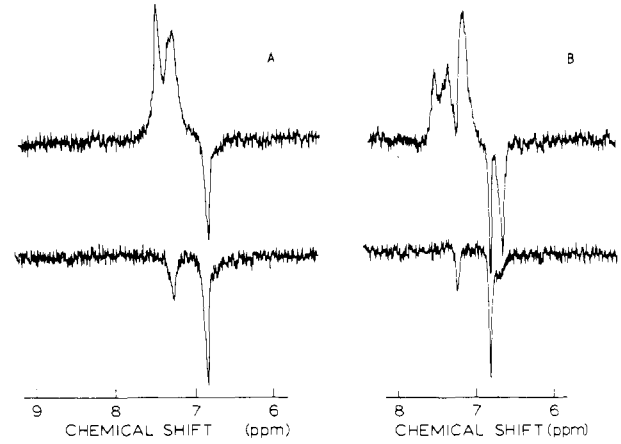

FIGURE 7: Photo-CIDNP spectra of porcine pancreatic phospholipase A_2 [upper curve in (A)] and prophospholipase A_2 [upper curve in (B)] and of a 1:2 molar mixture of PA_2 [lower curve in (A)] or the zymogen [lower curve in (B)] and sodium *n*-dodecyl sulfate at pH 6.0.

previously (de Araujo et al., 1979). In favorable cases such analysis of the UV difference absorbance or fluorescence data yield values for the dissociation constant K_D of the detergent–protein complex, for the number N of detergent monomers per protein molecule, and for the maximal optical signal C. Table IA shows a few results of such analyses for the first step of SDS binding to the active enzyme at pH 6.0 and 8.0. In Table IB some results are shown for the zymogen–SDS interaction. Although quantitative determination of binding parameters in the second step of the interaction process is impossible because of protein aggregation, the last column of Tables I gives approximate detergent concentrations required to completely transfer monomolecular protein into the high molecular weight aggregates. The first step of the binding process involving high-affinity binding of two detergent monomers to the protein surface is independent of Ca^{2+} ions. However, Ca^{2+} ions seem to shift the equlibrium of monomeric protein vs. protein aggregate toward the latter. Analysis of SDS binding to the zymogen gave comparable results. The increase in fluorescence quantum yields at pH 6.0 and 8.0 in the presence of Ca^{2+} ions indicated for the first binding step N values of about 2 and K_D values about 3 times higher than the corresponding values for the active enzyme. It is, however, clear that the tendency of the zymogen to aggregate into the high molecular weight complex is less than that of the active enzyme, and higher detergent concentrations are needed especially at pH 8.0.

The complete perturbation of Trp^3 in the second step of the process was used to estimate the time scale in which aggregation of protein and detergent molecules takes place. Preliminary experiments using porcine PA_2 and SDS showed half-times of about 300 ms to build up the high molecular weight complex. The first step, binding of two SDS monomers to the protein, appeared to be too fast to follow by using this technique.

Calorimetry. Heats of reaction of porcine pancreatic PA_2 and its zymogen at 15, 25, and 35 °C are shown in Figure 6 as a function of the SDS concentration. For both proteins the two-step interaction is clearly seen. Up to about 130 μM SDS PA_2 shows a small exothermic heat effect which is invariant with temperature. A stronger enthalpy change is generated in the second step, which is temperature dependent. Calculation of the heat capacity at 25 °C from the maximal enthalpy changes according to Donnér et al. (1976), a value of $\Delta C_p =$ -435 cal·K^{-1}·(mol of PA_2)$^{-1}$ is obtained. The zymogen does not show a heat effect in the first step. In the second step an exothermic heat effect is seen, which is strongly temperature dependent and gives rise to a ΔC_p value at 25 °C of −930 cal·K^{-1}·(mol of zymogen)$^{-1}$. The SDS concentration initiating the second step is about 2–3 times higher for the zymogen than for the active enzyme.

Photo-CIDNP Difference Spectra. Figure 7A (upper spectrum) shows the aromatic region of the photo-CIDNP difference spectrum for porcine PA_2 as such. The absorptive (upward) resonances arise from Trp^3 ring protons, and the emissive (downward) resonance arises from the Tyr^{123} 3,5-ring-H resonance at 6.80 ppm (Jansen et al., 1978).

Upon addition of a 2-fold molar excess of SDDS (Figure 7A, lower spectrum) the CIDNP signals of Trp^3 disappear. The two remaining sharp emissive lines at 7.26 and 6.80 ppm result from Tyr^{123} 2,6-H and 3,5-H resonances, respectively.

The same type of experiment carried out on porcine prophospholipase A_2 is shown in Figure 7B. In the upper spectrum (zymogen without added detergent) the absorptive lines stem again from the Trp^3 ring H's. The two sharp emissive lines at 6.80 and 6.69 ppm result from Tyr^{123} and Tyr^{69} 3,5-ring-H resonances, respectively. Addition of SDDS to the zymogen leads again to the disappearance of the Trp^3 CIDNP signals and most of the Tyr^{69} resonances. At least a 2-fold molar excess of SDDS is needed to give the maximum effect for Trp^3 under the conditions used. The two remaining emissive lines stem again from Tyr^{123} 2,6-H and 3,5-H resonances.

Enzyme Inactivation. The perturbation of Trp^3 in the first high-affinity binding step of anionic detergents clearly indicates that at least one of the first decyl sulfate molecules binds to the hydrophobic IRS. In order to find out whether the following decyl sulfate monomer is bound to the active site of the enzyme, irreversible inhibition using phenacyl bromide was studied according to Volwerk et al. (1974) in the presence of increasing concentrations of anionic detergents.

Sodium *n*-decyl sulfate was shown indeed to be able to protect the active site of the enzyme, however, *millimolar* concentrations of detergent were required. Analysis of the inhibition results according to Scrutton & Utter (1965) yielded a dissociation constant for the PA_2 active site–SDS complex of 2.5 mM at pH 6.4. As the high-affinity binding of detergents followed by enzyme aggregation described in this paper is characterized by binding constants in the *micromolar* range (cf. Table I), we must conclude that the enzyme aggregation at very low detergent concentration is induced by

the presence of about two firmly bound detergent monomers to the interface recognition site.

Discussion

Electrically neutral, single-chain detergent molecules such as lysolecithins and *n*-alkylphosphocholines have been extensively used in direct binding studies of phospholipase A_2 [for review see Volwerk & de Haas (1982); Teshima et al., 1981; Roberts et al., 1979]. These nondegradable substrate analogues form optically clear aqueous solutions characterized by cmc values varying from 1 μM (*n*-octadecylphosphocholine) to 10 mM (*n*-decylphosphocholine). The shorter chain homologues (C_{10}, C_{12}) allowed direct binding studies with molecularly dispersed lipids while interaction with micellar lipids could be studied conveniently by using the C_{16}/C_{18} derivatives.

For the porcine pancreatic PA_2 and its precursor it has been demonstrated that below the cmc of the *zwitterionic* substrate analogue both proteins bind only one lipid monomer per protein molecule. Protection studies involving irreversible active site inhibitors showed that the substrate analogue is bound to the active site in both the zymogen and the active enzyme (Volwerk et al., 1974). By use of different *n*-alkyl chain lengths it could be shown that this monomer binding to the active site is mainly hydrophobic in nature, that the microenvironment of Trp^3 is not directly perturbed, and that both proteins bind to the C_{10} homologue (*n*-decylphosphocholine) with dissociation constants of about 2 mM at pH 6.0 (Volwerk et al., 1974; van Dam-Mieras et al., 1975).

Above the cmc's of the *zwitterionic* substrate analogues the binding characteristics change: the active enzyme recognizes the micellar lipid–water interface and forms rather defined lipid–protein complexes consisting of 2 or 3 enzyme molecules and 30–50 lipid monomers per enzyme molecule. This interaction is characterized by a hydrophobic Trp perturbation resulting in a large increase in fluorescence quantum yield and a considerable blue shift of the emission maximum. The zymogen, however, due to its distorted lipid binding site, does not interact with these zwitterionic micellar surfaces, and no Trp perturbation is observed. The importance of these differences in binding characteristics between PA_2 and its zymogen is reflected in tremendous differences in catalytic turnover numbers for zwitterionic substrates above their cmc (Pieterson et al., 1974).

From the results described in the present study it is clear that substitution of the *neutral* phosphocholine moiety in *n*-decylphosphocholine by the *negatively charged* sulfate group has a drastic influence upon the binding properties to pancreatic PA_2. While (pro)phospholipase A_2 binding of a single zwitterionic detergent molecule takes place at the active site of the proteins and only in the presence of *millimolar* concentrations of detergent, the negatively charged alkyl sulfates with identical acyl chain lengths possess much higher affinities. Already at *micromolar* concentrations two alkyl sulfate monomers bind to the enzyme surface. This result strongly indicates that positively charged amino acid side chains on the enzyme surface must contribute to a large extent to the improved affinity. Also the invariance of ΔH with temperature as shown in the first binding step in Figure 6 points to charge interactions. Finally the strong pH effect upon detergent binding (Table I) is in agreement with a supposed ionic interaction between the negatively charged detergent monomers and surface-located positive side chains of the enzyme. The various spectroscopic data and in particular the photo-CIDNP spectra showed a rather strong perturbation of Trp^3 for both enzymes and of Tyr^{69} for the zymogen upon binding of two detergent monomers. For phospholipase A_2 the CIDNP signal of Tyr^{69} is not visible under the conditions used. Both Trp^3 and Tyr^{69} belong to the IRS (Verheij et al., 1981), which indicates that binding of at least one of the two detergent monomers takes place at the exposed hydrophobic surface patch of the enzyme molecules.

In contrast to several venom phospholipases A_2 (Smith & Wells, 1981; Roberts et al., 1977; Heinrikson et al., 1977; Verheij et al., 1981), the pancreatic enzymes show little tendency to dimerize in aqueous solution. However, not only will the binding of two alkyl sulfate molecules at the surface of the protein neutralize postively charged and exposed amino acid side chains but also at the same time the apolar character of the IRS will be strengthened. This results in a second, highly cooperative aggregation process which has shown to be rather fast and in which several enzyme molecules form a high molecular weight complex, including about 30 additional detergent molecules most probably in a pseudomicellar core. As can be derived from Figure 6, this aggregation process is accompanied by a large negative heat capacity change which is in the order of 2600 $cal \cdot K^{-1} \cdot mol^{-1}$ for PA_2 (six enzyme molecules in the complex) and 3800 $cal \cdot K^{-1} \cdot mol^{-1}$ for the zymogen (four enzyme molecules in the complex; see below). Such high values point to protein–protein interaction in the aggregate and possibly to conformational changes in the proteins. It should be realized that the formation and probably inclusion of a pseudomicellar detergent core take place at concentrations far below the cmc of the detergent. Although formation of very small SDDS aggregates at detergent concentrations below the cmc has been evoked in the literature (Nikolov et al., 1981; Panicheva & Markina, 1982), no lipid–water interfaces will be present.

Both active PA_2 and its zymogen are able to form high molecular weight protein–detergent complexes. Somewhat higher detergent concentrations are required for the zymogen, and the molecular weight of the final complex ($\sim$70 000) seems to be lower than that of the active PA_2 ($\sim$90 000). This indicates a different composition of both complexes. When the identical detergent to protein molar ratios found in the complexes formed with PA_2 and the zymogen are taken into account, it seems probable that although the mechanisms by which the aggregates are formed are rather similar in the zymogen–detergent complexes, only four protein molecules are built in. Also the ultraviolet and fluorescence spectroscopic data of the two-step interaction process with *n*-alkyl sulfates clearly demonstrate differences in the perturbation of aromatic surface residues in both proteins. In the following paper (Hille et al., 1983) it will be shown indeed that the aggregation processes described in the present study give rise to superactivation of the active PA_2 but not of the zymogen.

Registry No. SDS, 142-87-0; SDDS, 151-21-3; PA_2, 9001-84-7; prophospholipase A_2, 37350-21-3.

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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.

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