Interaction of Pancreatic Phospholipases A_2 and Semisynthetic Mutants with Anionic Substrates and Substrate Analogues†

Gerard H. de Haas,* Gustaaf J. M. van Scharrenburg,‡ and Arend J. Slotboom*

Laboratory of Biochemistry, State University of Utrecht, Transitorium III, University Centre "De Uithof", Padualaan 8, NL-3584 CH Utrecht, The Netherlands

Received November 7, 1986; Revised Manuscript Received February 5, 1987

ABSTRACT: Recently it was shown that porcine pancreatic phospholipase A_2 aggregates in the presence of submicellar concentrations of anionic substrates [van Oort, M. G., Dijkman, R., Hille, J. D. R., & de Haas, G. H. (1985) *Biochemistry 24*, 7993–7999]. In the resulting complexes the enzyme displays very high catalytic activity. In this study the interaction process was further investigated by using pancreatic phospholipases A_2 of different origins and several semisynthetic mutants in which one particular amino acid residue was substituted. By use of directing binding studies with a nondegradable anionic substrate analogue and monomolecular surface film kinetics on 1,2-didecanoyl-*sn*-glycerol 3-sulfate, it is shown that the aggregation process is controlled by the ionization state of the side chains of the amino acid residues at positions 6 and 17.

Mammalian pancreatic phospholipase A_2 (PLA)[1] (EC 3.1.1.4) catalyzes the hydrolysis of the 2-acyl ester bond in all types of 3-*sn*-phosphoglycerides. 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. Also, in the absence of bile salts the pancreatic enzymes are known to attack anionic phospholipids preferentially (Van Deenen & de Haas, 1963; Hendrickson et al., 1981). The best substrate known today for pancreatic phospholipases A_2, 1,2-dioctanoyl-*sn*-glycerol 3-sulfate, is hydrolyzed an order of magnitude faster than the corresponding lecithin (Van Oort et al., 1985b). Recently we demonstrated that for the diacylglycerosulfates maximal enzyme activity of porcine PLA is found in premicellar multienzyme complexes of high molecular weight containing a number of substrate monomers (Van Oort et al., 1985a,b). The formation of these complexes has been tentatively explained as follows. The high-resolution X-ray structures of pancreatic PLAs show that the active-site cleft is surrouned by a collar of hydrophobic amino acid side chains located at the surface of the enzyme (Dijkstra et al., 1981, 1983). Through this so-called lipid binding domain the enzyme anchors itself to organized lipid–water interfaces. The X-ray model of the porcine PLA shows, in addition, that the lipid binding domain contains also a cluster of positively charged side chains at positions 6, 10, and 17. This cationic center will counteract the tendency of the enzyme to aggregate. It has been proposed that in the presence of submicellar concentrations of negatively charged substrates a few lipid monomers bind with high affinity to the lipid binding domain, thereby neutralizing the positive charges and increasing the surface hydrophobicity of the protein. The resulting change in the hydrophobic–hydrophilic balance leads to a second binding process in which protein aggregation occurs. This protein aggregation is accompanied and stabilized by a concomitant gathering of substrate molecules at lipid concentrations well below the cmc and results in higher molecular weight enzyme–substrate complexes consisting of several PLA molecules and about 30–40 lipid monomers.

It is the purpose of this study to gain further insight in the formation of these premicellar E–S complexes and to obtain additional support for the role of certain positively charged amino acid side chains in the aggregation process with both native enzymes and semisynthetic mutants. From direct binding studies and monolayer kinetics it is concluded that the ionization state of the side chains of the amino acid residues at positions 6 and 17 influences to a large extent the aggregation process.

Materials and Methods

Porcine prophospholipase A_2, porcine isoprophospholipase A_2, and bovine prophospholipase A_2 were purified and converted into their corresponding phospholipases A_2 as described previously [Nieuwenhuizen et al. (1974), Van Wezel and de Haas (1975), and Dutihl et al. (1975), respectively]. The pancreatic phospholipases A_2 were fully ϵ-amidinated according to the procedure of Wallace and Harris (1984), purified, and converted into the corresponding ϵ-amidinated phospholipases A_2 (AMPAs) as described (Slotboom & De Haas, 1975; Van Scharrenburg et al., 1981; Van Scharrenburg et al., 1984b). Porcine [Phe^3]AMPA was prepared as described before (Slotboom & de Haas, 1975). Bovine [Arg^6]AMPA and porcine [Ala^7]AMPA were prepared as described by Van Scharrenburg et al. (1981 and 1983, respectively). Porcine des-(Ala^1–Ser^7)-AMPA was prepared and purified as described previously (Van Scharrenburg et al., 1983). Porcine des-(Ala^1–Ser^7)-iso-AMPA was prepared similarly as described for the bovine fragment (Van Schar-

† These investigations were carried out under the auspices of The Netherlands Foundation for Chemical Research (SON) and with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

* Address correspondence to these authors.

‡ Present address: Duphar BV, 1380 AA Weesp, The Netherlands.

[1] Abbreviations: PLA, phospholipase A_2; AMPREC, fully ϵ-amidinated prophospholipase A_2; AMPA, fully ϵ-amidinated phospholipase A_2; Pipes, 1,4-piperazinediethanesulfonic acid; Bu^t, *tert*-butyl; Boc, *tert*-butyloxycarbonyl; FMOC, *N*-9-fluorenylmethoxycarbonyl; For, formyl; LC, liquid chromatography; DEAE, diethylaminoethyl; CM, carboxymethyl; *rac*-DHGS, *rac*-1,2-(diheptylcarbamoyl)glycerol 3-sulfate; TFA, trifluoroacetic acid; NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; cmc, critical micelle concentration.

0006-2960/87/0426-3402$01.50/0

renburg et al., 1981). N^{α}-Boc-Ala-Leu-Trp(For)-Gln-Phe-Asn-Gly was prepared by solid-phase peptide synthesis and purified as described before (Van Scharrenburg et al., 1983). *rac*-1,2-(Diheptylcarbamoyl)glycerol was synthesized according to the method of Gupta and Bali (1981) and converted into *rac*-1,2-(diheptylcarbamoyl)glycerol 3-sulfate by established procedures (Van Oort et al., 1985b). 1,2-Didodecanoyl-*sn*-glycerol 3-sulfate was prepared as described previously (Van Oort et al., 1985b). Methyl acetamidate hydrochloride was prepared according to the procedure of Hunter and Ludwig (1962). *N*-α-FMOC-amino acids were purchased from Chemalog, Chemical Dynamics Corp. (South Plainfield, NJ), and N^{α}-Boc-Ala was from Vega (Tucson, AZ). DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were purchased from Whatman (Maidstone, England), and various Sephadex products were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). They were prepared for use according to the manufacturer's recommendations. All other chemicals used were of the highest purity available.

Preparation of Porcine [Glu⁶,Ala⁷]AMPA. *N*-BocAla was esterified to chloromethylated polystyrene resin that had been 1% cross-linked with divinylbenzene (1.34 mmol of Cl/g of resin) according to the procedure of Gisin (1973). After removal of the Boc group with 50% TFA in CH_2Cl_2, the peptide H_2NAla-Leu-Trp-Gln-Phe-Glu(OBu^t)-Ala was prepared by solid-phase peptide synthesis using FMOC for α-NH_2 protection and 50% piperidine in CH_2Cl_2 for deprotection (Van Scharrenburg et al., 1984a). FMOC-protected amino acids were coupled as the symmetrical anhydrides, prepared "in situ" according to the procedure of Meienhofer (1979). Cleavage of the peptide from the resin was done by catalytic transfer hydrogenation using palladium acetate and ammonium formate as described by Anwer et al. (1983). The peptide was extracted from the resin with acetic acid–H_2O (1:1 v/v), lyophilized, and purified by LC on a C_{18} reversed-phase Lichrosorb column using a gradient from 10 to 90% acetonitrile in H_2O. The pure peptide was then reacted with di-*tert*-butyl dicarbonate (Moroder et al., 1976) to protect the α-NH_2 group and subsequently purified as described above. Coupling of the pure N^{α}-Boc-Ala-Leu-Trp-Gln-Phe-Glu(OBu^t)-Ala to porcine des-(Ala¹–Ser⁷)-AMPA was performed according to the mixed carbonic anhydride procedure of Meienhofer (1979). After H_2NOH treatment (1 M, pH 7.0) at room temperature, the reaction mixture was dialyzed and lyophilized. The protected protein was purified by ion-exchange chromatography on CM-cellulose at pH 5. Fractions containing the protected AMPA analogue were pooled, dialyzed, and lyophilized. Removal of the Boc and OBu^t groups was achieved by treatment with TFA. After lyophilization, pure porcine [Glu⁶-Ala⁷]AMPA was obtained by chromatography on DEAE-cellulose at pH 7.5.

Preparation of Porcine [Asn⁶,Gly⁷]iso-AMPA. This analogue was prepared by coupling of N^{α}-Boc-Ala-Leu-Trp-(For)-Gln-Phe-Asn-Gly to porcine des-(Ala¹–Ser⁷)-iso-AMPA as described previously (Van Scharrenburg et al., 1983). After purification by chromatography on DEAE-cellulose and CM-cellulose, pure [Asn⁶,Gly⁷]iso-AMPA was obtained. The enzymatic properties of all ϵ-amidinated phospholipases A_2 and the semisynthetic mutants are compared in Table I.

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

Table I: Kinetic Properties of Fully ϵ-Amidinated Porcine Phospholipase A_2, Porcine Isophospholipase A_2, and Bovine Phospholipase A_2 (AMPAs) and of Various Semisynthetic Mutant AMPAs

	sp act.[a] (egg yolk assay) [μequiv min^{-1} (mg of protein)$^{-1}$]	V_{max}[b] (micellar L-dioctanoyllecthin assay) [μequiv min^{-1} (mg of protein)$^{-1}$]
porcine		
AMPA	330	2200
[Ala⁷]AMPA	250	1500
[Glu⁶,Ala⁷]AMPA[c]	30	500
[Phe³]AMPA	105	860
porcine-iso		
AMPA	120	2050
[Asn⁶,Gly⁷]AMPA[c]	70	1400
bovine		
AMPA	50	2300
[Arg⁶]AMPA	120	6300

[a] Determined at pH 8 as described previously (Van Scharrenburg et al., 1982). [b] Determined by titration of fatty acid liberated with 10.3 mM NaOH at pH 6 and 45 °C (50 mM $CaCl_2$, 10 mM acetate, and 0.1 M NaCl) as described previously (Van Scharrenburg et al., 1981). [c] In these mutants, Ser⁷ was substituted by Ala and Gly, respectively, because coupling yields of N-terminal heptapeptides are much higher when Ala or Gly are C-terminal as compared to Ser (Van Scharrenburg et al., 1983). [Ala⁷]AMPA shows that these substitutions have only a small effect on catalytic properties.

Monolayer Experiments. The kinetic experiments were performed with the "zero-order trough" (Verger & De Haas, 1973) containing two compartments, a reaction compartment and a reservoir compartment connected by a small surface channel. Enzyme was injected under the film in the reaction compartment only, whereas the substrate film covers both compartments. The reservoir compartment contains a mobile barrier, which was used to correct for substrate molecules removed from the film in the reaction compartment by enzymatic hydrolysis, thereby keeping the surface pressure (π) constant. The latter is measured in the reservoir compartment with a Wilhelmy plate (perimeter 3.94 cm) attached to a Beckman R II C Model LM 600 electromicrobalance that in turn is connected to a microprocessor which controls the movement of the mobile barrier. In the magnetically stirred reaction compartment the temperature was controlled with a circulating water bath at 25 °C. During the monolayer experiment, data (bar positions) are stored every 10 s into the internal memory of the microprocessor. After the experiment, data can be stored on diskettes for further analysis (Donné-Op den Kelder et al., 1984). The analysis of the kinetic data was performed according to the method described by Verger et al. (1973). A program written in BASIC for nonlinear regression analysis based on the method of Fletcher and Powell (1963) was used. The unweighted kinetic data were fitted to the equation $P = V_m t + V_m t(e^{-t/\tau} - 1)$ where P is the bar position (mm) at time t (min), V_m is the enzymatic velocity under steady-state conditions, and τ is the induction time of the process, which reflects penetration of the enzyme into the monolayer. From the above equation V_m can be deduced to be the slope of the titration curve when time approaches infinity, i.e., the asymptotic value ($t \gg \tau$). Extrapolation of this asymptote to the x axis gives the value for τ.

Protein concentrations were determined from the absorbances at 280 nm with an $E_{1\,cm}^{1\%}$ of 13.0 for ϵ-amidinated porcine phospholipase A_2, porcine isophospholipase A_2, bovine ϵ-amidinated phospholipase A_2, and their analogues. For

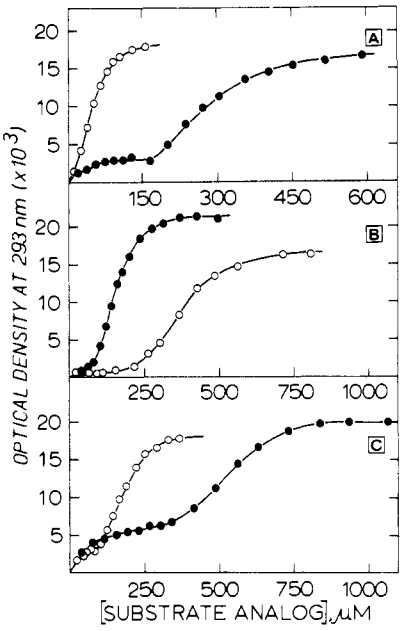

FIGURE 1: Optical density at 293 nm from ultraviolet absorbance difference spectra at various concentrations of substrate analogue [*rac*-1,2-(diheptylcarbamoyl)glycerol 3-sulfate]. (A) Porcine AMPA (○) and porcine [Glu^6,Ala^7]AMPA (●), initial protein concentration 23.8 μM. (B) Porcine iso-AMPA (●) and porcine [Asn^6,Gly^7]iso-AMPA (○), initial protein concentration 20.6 μM. (C) bovine AMPA (●) and bovine [Arg^6]AMPA (○), initial protein concentration 25.8 μM. Conditions: 10 mM Pipes, pH 6.0; T = 25 °C.

porcine des-(Ala^1–Ser^7)-AMPA and iso-AMPA a value of 8.8 was used.

RESULTS

Recently we studied the interaction between pancreatic phospholipase A_2 and the nondegradable substrate analogue *rac*-1,2-(diheptylcarbamoyl)glycerol 3-sulfate (*rac*-DHGS) using ultraviolet difference spectroscopy (Van Oort et al., 1985b). Upon titration of the enzyme with increasing amounts of detergent, all well below the cmc (=1.6 mM), we found from the intensity of the difference maxima and minima that the binding process is biphasic. The first process, characterized by maximal ultraviolet difference absorption at 290 nm, is saturated at a detergent concentration of about 300 μM for the bovine AMPA. This interaction is followed by a second binding process also reaching saturation (at about 950 μM for the bovine AMPA) and characterized by maximal difference absorption at 293 nm. The biphasic character of the interaction with bovine AMPA is clearly seen in Figure 1C (right curve), where the optical density at 293 nm is plotted as a function of substrate analogue concentration. In order to find out whether the side chain of the amino acid at position 6 is involved in this interaction phenomenon, similar titrations were performed with three pairs of enzymes, each consisting of a "native" enzyme and a semisynthetic mutant differing in the amino acid present in position 6. (See Table II.) The results of these ultraviolet difference titrations are given in Figure 1. Although all six proteins show a biphasic binding behavior, this is most pronounced for the porcine enzyme in which Arg^6 is replaced by Glu (panel A, right curve) and for the porcine isoenzyme in which Arg^6 is replaced by Asn (panel B, right curve). Substitution of Asn^6 in bovine AMPA by Arg (panel C) results in considerably improved binding both in the first and second processes. These results strongly suggest that Arg^6 is involved both in the initial high-affinity binding of a few glycerol sulfate monomers (first binding process) and in the formation of high molecular weight enzyme–detergent complexes (second binding process).

Table II: N-Terminal Amino Acid Sequences of Fully ε-Amidinated Porcine Phospholipase A_2, Porcine Isophospholipase A_2, and Bovine Phospholipase A_2 (AMPAs) and of Three Semisynthetic Mutant AMPAs in Which the Amino Acid in Position 6 is Replaced by Glu, Asn, and Arg, Respectively[a]

enzyme	position 1	6	10[b]	17
porcine AMPA	Ala ...	Arg ...	Lys ...	His
porcine mutant AMPA	Ala ...	Glu ...	Lys ...	His
porcine iso-AMPA	Ala ...	Arg ...	Lys ...	Asp
porcine mutant iso-AMPA	Ala ...	Asn ...	Lys ...	Asp
bovine AMPA	Ala ...	Asn ...	Lys ...	Glu
bovine mutant AMPA	Ala ...	Arg ...	Lys ...	Glu

[a] For the complete amino acid sequences the reader is referred to Volwerk and de Haas (1982) and Slotboom et al. (1982). [b] Lys stands for ε-amidino-Lys.

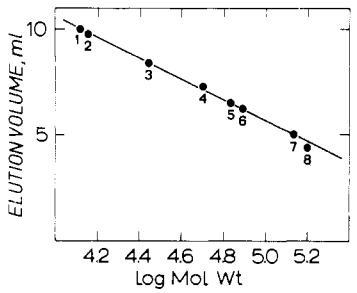

FIGURE 2: Relationship between elution volume and molecular weight of various phospholipases A_2 and mutants in the presence of *rac*-1,2-(diheptylcarbamoyl)glycerol 3-sulfate on a Sephadex G-100 fine column (70 × 0.7 cm) at 25 °C and pH 6 (10 mM Pipes). Calibration proteins, ribonuclease (1), M_r 13 000, staphylococcal protease (3), M_r 27 700, porcine lipase (4), M_r 50 000, albumin (5), M_r 68 000, albumin dimer (7), M_r 136 000, and ferritin (8), M_r 450 000, were eluted after the column was equilibrated with 130 μM *rac*-1,2-(diheptylcarbamoyl)glycerol 3-sulfate. 2 = bovine AMPA at 250 μM *rac*-DHGS (M_r 14 000) and porcine [Glu^6,Ala^7]AMPA at 130 μM *rac*-DHGS (M_r 14 000); 6 = porcine AMPA at 130 μM *rac*-DHGS (M_r 75 000) and bovine [Arg^6]AMPA at 250 μM *rac*-DHGS (M_r 75 000).

The difference in molecular weight of the complexes formed during the first binding step and the second interaction in which enzyme aggregation takes place is shown in Figure 2 by using gel filtration on a calibrated Sephadex G-100 column. From the spectral results (Figure 1, panel A) it is obvious that, at 130 μM *rac*-DHGS, porcine AMPA is completely converted into the premicellar aggregate, whereas the mutant [Glu^6,Ala^7]AMPA is still in the first binding phase. The elution profile of both proteins on the Sephadex column equilibrated with 130 μM *rac*-DHGS is in agreement: the mutant enzyme elutes at a position corresponding to a molecular weight of 14 000 (monomeric protein), porcine AMPA gives a molecular weight of 75 000. A similar behavior is observed for the bovine AMPA and its Arg^6 mutant (Figure 1, panel C). Elution of the complexes formed in 250 μM *rac*-DHGS showed a monomeric protein for AMPA (molecular weight 14 000), whereas the Arg^6 mutant is fully aggregated (molecular weight 75 000) at this detergent concentration.

Inspection of the 1.7-Å resolution X-ray model of the bovine pancreatic enzyme reveals that besides the side chain of the amino acid in position 6, the residues in positions 10 and 17 are located in the heart of the lipid binding domain. From Table II it is clear that at position 10 an invariant Lys residue is present that cannot be substituted easily by semisynthesis.

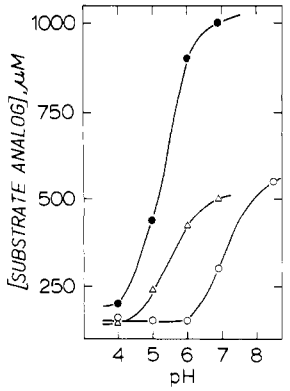

FIGURE 3: Plot showing the extent of aggregation of various AMPAs and substrate analogue (*rac*-DHGS) vs. concentration of substrate analogue at different pH values (cf. Figure 2). Bovine AMPA (●), initial protein concentration 25.8 μM; porcine iso-AMPA (△), initial protein concentration 20.6 μM; porcine AMPA (○), initial protein concentration 23.8 μM. Conditions: pH 4 and 5, 10 mM sodium acetate; pH 6 and 6.9, 10 mM Pipes; pH 8.5, 10 mM Tris; T = 25 °C.

Much more interesting is nature's variation in position 17, especially when we compare the porcine PLA with its isoenzyme. Both proteins differ in only four amino acid residues, and only two substitutions involve charged residues. His^{17} in the porcine enzyme is replaced by Asp in the isoenzyme, and Glu^{71} in the porcine enzyme becomes Asn in the isoenzyme. From ultraviolet difference titrations as shown in Figure 1, it is possible to derive approximately the minimal substrate analogue concentration needed to convert all enzyme in its high molecular weight aggregated form.

Figure 3 records these detergent concentrations at various pH values for the three AMPAs from porcine and bovine pancreas. It is clear that all three enzymes gradually lose their tendency to aggregate at higher pH values, and increasingly higher *rac*-DHGS concentrations are required for complete complexation. From the shapes of the curves it is tempting to propose that the ionization behavior of a single group is responsible for the change in aggregation behavior of the various AMPAs. Taking into account the above-mentioned minimal difference between the porcine enzyme and isoenzyme (His^{17} → Asp), we suggest that deprotonation of the side chain at position 17 strongly influences the tendency to form high molecular weight complexes and that these amino acids possess pK values of about 5.5 (Glu and Asp in bovine and porcine iso-AMPAs, respectively) and 7 (His in porcine AMPA).

In order to obtain additional information about hydrophobic amino acid side chains involved in the aggregation process of phospholipase A_2 with anionic substrate analogues at submicellar concentrations, porcine AMPA and a semisynthetic mutant in which the single Trp^3 was replaced by Phe were titrated with *rac*-DHGS. Figure 4 shows the maximal ultraviolet difference signals for both proteins upon complete aggregation at pH 6 and 8.5. From previous work (Meijer et al., 1979) we know that out of the eight tyrosine residues of porcine PLA, only Tyr^{69} is located in the lipid binding domain and involved in the interaction of the enzyme with organized lipid–water interfaces. Therefore, we attribute the pure tyrosine difference spectrum (Figure 4, curve D) of the Phe^3 mutant to hydrophobic perturbation of Tyr^{69} during the premicellar aggregation process. Curve C in Figure 4 shows the same tyrosine perturbation of the Phe^3 mutant now at pH 8.5. Compared to the difference spectrum at pH 6 (Figure 4, curve D) the difference spectrum at pH 8.5 (Figure 4, curve C) shows an increase as well as a small blue shift of the difference peak at about 290 nm and the appearance of a broad

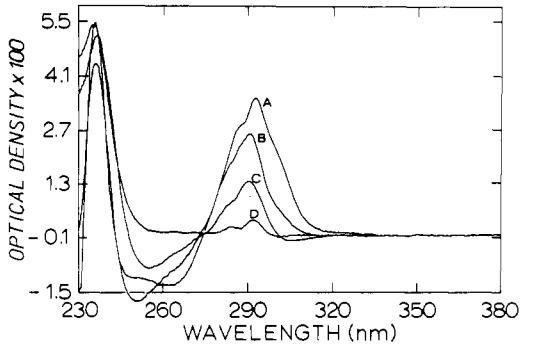

FIGURE 4: Ultraviolet absorbance difference spectra produced by the interaction of various pancreatic phospholipases A_2 and mutants at saturating concentrations of *rac*-1,2-(diheptylcarbamoyl)glycerol 3-sulfate (*rac*-DHGS): A, porcine AMPA (33.4 μM) and *rac*-DHGS (381 μM), pH 6; B, porcine AMPA (34.2 μM) and *rac*-DHGS (877 μM), pH 8.5; C, porcine [Phe^3,Ala^7]AMPA (34 μM) and *rac*-DHGS (683 μM), pH 8.5; D, porcine [Phe^3,Ala^7]AMPA (33.4 μM) and *rac*-DHGS (183.5 μM), pH 6. Conditions: pH 6, 10 mM Pipes; pH 8.5, 10 mM borate; T = 25 °C.

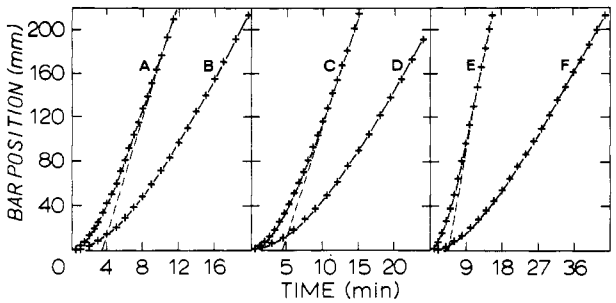

FIGURE 5: Kinetics of the hydrolysis of a 1,2-didecanoyl-*sn*-glycerol 3-sulfate film at 30 dyn/cm upon injection of various AMPAs and semisynthetic mutants. The curves represent the results of the computer fit, and the points on these curves represent the experimentally obtained data points. The slope of the asymptotes (dotted lines) to the curves A, C, and E represent the steady-state velocity (V_m). Extrapolation of this asymptote to the x axis gives the value for the induction time (τ). In the case of rapidly penetrating enzymes (short τ), the lag time can be measured manually. Slowly penetrating enzymes (curves B, D, and F) require the use of the computer in order to obtain accurate τ values. A, bovine [Arg^6]AMPA; B, bovine AMPA; C, porcine iso-AMPA; D, porcine [Asn^6,Gly^7]iso-AMPA; E, porcine [Ala^7]AMPA; F, porcine [Glu^6,Ala^7]AMPA. Conditions: 10 mM Tris, 0.1 M NaCl, 20 mM $CaCl_2$, pH 8; T = 25 °C.

trough around 250 nm. It is well-known (Herskovits, 1967) that these spectral changes specifically reflect ionization of tyrosyl residues. The (partial) ionization of Tyr^{69} in the [Phe^3]AMPA mutant is in agreement with its low pK value found previously (unpublished experiments). Curves A and B (Figure 4) represent the difference spectra produced by porcine AMPA in the premicellar aggregation process at pH 6 and 8.5, respectively. Comparison of the difference spectra A and D shows that the difference peak at 293 nm in AMPA and the trough between 250 and 270 nm are caused mainly by hydrophobic perturbation of Trp^3. The lowering of the 293-nm peak in AMPA and the small blue shift upon raising the pH from 6 to 8.5 (curve B) are probably caused by quenching of the Trp absorbance upon ionization of Tyr^{69}. The penetrating power of the various enzymes and mutants in densely packed monomolecular surface layers was measured by determining the kinetics of hydrolysis of 1,2-didecanoyl-*sn*-glycerol 3-sulfate films as described previously (Donné-Op den Kelder et al., 1984) (Figure 5). With this technique accelerating kinetics are observed under certain conditions and long induction times are present. This is caused by a slow reversible penetration of the enzyme into the monolayer. These lag times τ, which are dependent on enzyme species, chemical

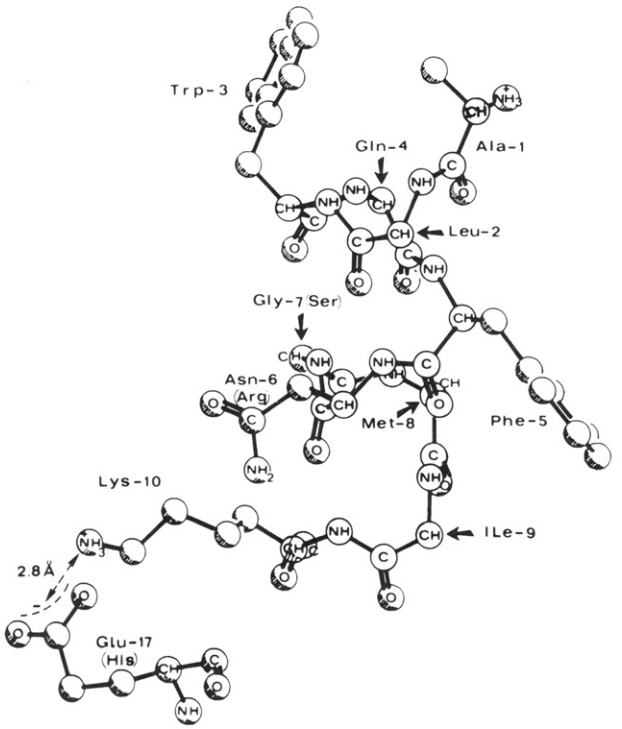

FIGURE 6: Structure of bovine pancreatic phospholipase A_2. Computer drawing representing the N-terminal α-helical structure of bovine pancreatic phospholipase A_2 and the hydrogen bond between the ϵ-NH_3^+ of Lys^{10} and the COO^- of Glu^{17}. For the sake of clarity only the side chains of Ala^1, Trp^3, Asn^6, Lys^{10}, and Glu^{17} are shown. In parentheses the corresponding amino acid residues in porcine pancreatic phospholipase A_2 are given.

structure of the substrate, and surface pressure of the monolayer, characterize the penetrating power of the protein.

Discussion

Chemical modifications and semisynthetic studies on pancreatic phospholipases A_2 have shown that the aromatic amino acid residues Trp^3 and Tyr^{69} are directly involved in the hydrophobic interaction of the enzyme with neutral lipid–water interfaces (Volwerk & de Haas, 1982).

From this study it is clear that the same residues are also perturbed in the premicellar high molecular weight detergent–protein aggregates formed upon interaction of the enzyme with negatively charged substrates. The ultraviolet difference spectra obtained upon interaction of pancreatic PLAs with very low concentrations of *rac*-DHGS are characteristic for hydrophobic perturbation of the single Trp^3 in these enzymes. In order to analyze these difference spectra in more detail, similar titrations were performed with a semisynthetic mutant of PLA in which the single Trp^3 was replaced by Phe. When this mutant was completely converted into the premicellar protein–detergent complex at pH 6, the ultraviolet difference spectrum D of Figure 4 was obtained. This is a pure Tyr perturbation spectrum, and the optical density difference value suggests that a single Tyr side chain is hydrophobically perturbed. A similar titration, now at pH 8.5, is represented by curve C in Figure 4. The position of the maximum and minimum difference peaks points to an advanced ionization of this tyrosine side chain, and its low pK value is in agreement with known properties of Tyr^{69} (unpublished experiments). The lipid binding domain appears, however, to be partly constituted by positively charged amino acid residues like Arg^6 and His^{17}, located in the N-terminal region of porcine PLA (Figure 6). In order to obtain more information about the possible role of these cationic side chains

Table III: Kinetic Parameters of the Hydrolysis of 1,2-Didecanoyl-*sn*-glycerol 3-Sulfate Monolayer by Various ϵ-Amidinated Phospholipases A_2 and Semisynthetic Mutants[a]

	lag time, τ (min), at		V_m [mm min^{-1} (mg of protein injected)$^{-1}$] at	
enzyme	10 dyn/cm	30 dyn/cm	10 dyn/cm	30 dyn/cm
porcine				
AMPA	4.5	4.0	14.4	23.8
[Ala^7]AMPA	4.5	4.5	13.3	22.0
[Glu^6,Ala^7]-AMPA	15.7	18.0	0.5	0.6
porcine-iso				
AMPA	3.4	4.1	8.2	13.5
[Asn^6,Gly^7]-AMPA	7.8	11.9	5.2	6.9
bovine				
AMPA	6.2	9.7	7.7	9.7
[Arg^6]AMPA	3.2	3.6	10.2	21.3

[a] For experimental conditions see Figure 5.

in the interaction with negatively charged substrate analogues, we compared the behavior of pancreatic PLAs of various origins and a number of semisynthetic mutants. Because site-specific substitution of a single amino acid by semisynthesis requires unreactive ϵ-amino groups, the native pancreatic phospholipases A_2 were converted into their fully ϵ-amidinated derivatives (AMPAs). These latter proteins have been shown previously to possess properties very similar to those of the native enzymes (Slotboom et al., 1982). Table I compares the various enzymes and mutants used in this study, and Table II shows the identity of the amino acid residues in positions 1, 6, 10, and 17 of the polypeptide chains. It is clear that all the semisynthetic mutants are enzymatically active both on mixed micelles of sodium deoxycholate and long-chain egg lecithin and on micellar dioctanoyllecithin, and that the presence of Arg in position 6 has a very favorable influence on the specific activity. Substitution of Asn^6 in the bovine enzyme by Arg results in 2–3 times higher k_{cat} values. On the other hand, replacement of Arg^6 in the porcine enzyme by the neutral Asn or the negatively charged Glu results in k_{cat} losses of about 40 and 80%, respectively. The same trend is observed by comparing the steady-state velocities (V_m) of hydrolysis of monomolecular surface films (Table III). It has to be kept in mind, however, that this latter technique yields only apparent velocities, because the amount of enzyme present in the substrate film is unknown (Verger et al., 1973). Especially the extremely low V_m found for the porcine [Glu^6,Ala^7]AMPA might be due to a much smaller amount of this enzyme present in the negatively charged substrate film as compared with the mutant enzymes containing Asn^6 or Arg^6. Amino acid substitutions in the 6-position not only influence k_{cat} but they also drastically change the binding and aggregation behavior of the mutants in the presence of anionic substrate analogues. As can be deduced from Figures 1 and 2, all enzymes containing Arg in position 6 eagerly bind a few anionic detergent molecules with retention of their low molecular weight (14000). At slightly higher detergent concentrations, but well below the cmc, this high affinity binding is followed by protein aggregation and formation of high molecular weight enzyme–detergent complexes (75000). Substitution of Arg^6 by Asn or Glu not only lowers the affinity for the anionic detergent in the first binding process, but at the same time the tendency of the enzyme to aggregate diminishes and considerably higher detergent concentrations are needed to transfer all protein into the high molecular weight aggregates.

The reduced affinity of pancreatic PLAs for anionic substrates upon replacement of Arg^6 by a neutral or negatively

charged amino acid is most evident from the changes in lag time during the pre-steady-state hydrolysis of monomolecular substrate films. Table III shows that even in high-pressure surface films (π = 30 dyn/cm), there is a rapid penetration of those enzymes and mutants containing Arg in position 6 (short τ values of about 4 min). Substitution of Arg^6 by Asn in the bovine and porcine iso-AMPAs results in delayed penetration of the enzymes characterized by τ values of 10–12 min. The presence of a negatively charged amino acid in position 6 as in the porcine [Glu^6,Ala^7]AMPA significantly obstructs interaction, and a τ value of 18 min is observed. It has to be remarked that, in contrast to the apparent V_m values in monolayer kinetics, the lag times, τ, are independent of enzyme concentration and yield valuable information on the penetration power of lipolytic enzymes (Verger et al., 1973).

The above results, obtained with submicellar concentrations of anionic substrates and substrate analogues, are in agreement with previous findings that the amino acid chain in position 6 of bovine and porcine pancreatic PLAs has a large influence on both binding and hydrolysis of micellar zwitterionic substrates (Volwerk & de Haas, 1982). Of course it might be argued that the porcine and bovine PLAs, notwithstanding their high sequence homology, contain several more substitutions outside position 6, which might influence the kinetic and aggregation behavior. For this very reason we included the porcine pancreatic isoenzyme. This minor isophospholipase A_2 of porcine pancreas differs from the main enzyme by four amino acid substitutions, only two of which involve charged residues: Ala^{12},His^{17},Met^{20}, and Glu^{71} in the porcine PLA by Thr^{12},Asp^{17},Leu^{20}, and Asn^{71} in the porcine iso-PLA, respectively. The great value of this isoenzyme lacking Glu^{71} has been demonstrated before in the localization of the second Ca^{2+}-binding site in porcine pancreatic PLA(Donnē-Op den Kelder et al., 1983). The pH-dependent aggregation of pancreatic enzymes in the presence of anionic substrate analogues, reported in Figure 3, demonstrates again the usefulness of closely related isoenzymes: the observation that the isoenzyme at pH 6 needs 3 times more anionic detergent for complete aggregation points to the important role of the ionization state of the side chain at position 17 in the aggregation process. The approximate pK values of Asp^{17} in iso-PLA and His^{17} in PLA, derived from these titration curves, are 5.5 and 7, respectively. This latter value is in reasonable agreement with the pK of His^{17} (6.6) in porcine PLA as determined by NMR (Aguiar et al., 1979).

The lipid binding domain of porcine pancreatic phospholipase A_2 contains, besides a number of apolar amino acid side chains, a cluster of positively charged amino acids in positions 6, 10, and 17 (Dijkstra et al., 1983). This excess of positively charged residues on the enzyme's surface suggests that salt bridge formation with anionic substrates and analogues is important in the formation of high molecular weight enzyme–detergent complexes. At pH 6 the affinity for anionic substrate analogues of porcine iso-AMPA and bovine AMPA decreases in this order as compared to that of porcine AMPA. This effect is explained by the fact that the net positive charge of this N-terminal polar cluster decreases from +3 (porcine PLA) to + 1 (porcine iso-PLA) to 0 (bovine PLA). Recently Forst et al. (1986) presented evidence that a similar cluster of basic amino acid residues at the N-terminus of PLA from *Agkistrodon halys blomhoffii* is important for the action of this PLA on *Escherichia coli* killed by the bactericidal protein of neutrophils.

ACKNOWLEDGMENTS

We thank Mr. R. Dijkman for synthesis and purification of the substrates and substrate analogues and W. Atsma for his help in some of the experiments. We are much indebted to Prof. Dr. G. Langer (University of California, Los Angeles) for carefully proofreading the manuscript.

REFERENCES

Aguiar, A., de Haas, G. H., Jansen, E. H. J. M., Slotboom, A. J., & Williams, R. J. P. (1979) *Eur. J. Biochem. 100*, 511–518.

Anwer, M. K., Spatola, A. F., Bossinger, C. D., Flanigan, E., Liu, R. C., Olson, D. B., & Stevenson, D. (1983) *J. Org. Chem. 48*, 3503–3507.

De Araujo, P. S., Rosseneu, M. Y., Kremer, J. M. H., Van Zoelen, E. J. J., & de Haas, G. H. (1979) *Biochemistry 18*, 580–586.

Dijkstra, B. W., Kalk, K. H., Hol, W. G. J., & Drenth, J. (1981) *J. Mol. Biol. 147*, 97–123.

Dijkstra, B. W., Renetseder, R., Kalk, K. H., & Drenth, J. (1983) *J. Mol. Biol. 168*, 163–179.

Donnē-Op den Kelder, G. M., de Haas, G. H., & Egmond, M. R. (1983) *Biochemistry 22*, 2470–2478.

Donnē-Op den Kelder, G. M., Van der Wildt, H., & de Haas, G. H. (1984) *Anal. Biochem. 142*, 126–133.

Dutihl, C. E., van Doren, P. J., Verheul, F. E. A. M., & de Haas, G. H. (1975) *Eur. J. Biochem. 53*, 91–97.

Fletcher, R., & Powell, M. J. D. (1963) *Comput. J. 6*, 163–173.

Forst, S., Weiss, J., Blackburn, P., Frangione, B., Goni, F., & Elsbach, P. (1986) *Biochemistry 25*, 4309–4314.

Gisin, B. F. (1973) *Helv. Chim. Acta 56*, 1476–1482.

Gupta, C. M., & Bali, A. (1981) *Biochim. Biophys. Acta 663*, 506–515.

Hendrickson, H. S., Trygstad, W. M., Loftness, T. L., & Sailor, S. L. (1981) *Arch. Biochem. Biophys. 212*, 508–514.

Herskovits, T. T. (1967) *Methods Enzymol. 11*, 748–775.

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

Hunter, M. J., & Ludwig, M. L. (1962) *J. Am. Chem. Soc. 84*, 3491–3504.

Meienhofer, J. (1979) in *The Peptides* (Gross, M., & Meienhofer, J., Eds.) Vol. 1, pp 263–314, Academic, London.

Meijer, H., Puijk, W. C., Dijkman, R., Foda-van der Hoorn, M. M. E. L., Pattus, F., Slotboom, A. J., & de Haas, G. H. (1979) *Biochemistry 18*, 3589–3597.

Moroder, L., Hallett, A., Wünsch, E., Keller, O., & Wersin, G. (1976) *Hoppe-Seyler's Z. Physiol. Chem. 357*, 1651–1653.

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

Slotboom, A. J., & de Haas, G. H. (1975) *Biochemistry 14*, 5394–5399.

Slotboom, A. J., Verheij, H. M., & de Haas, G. H. (1982) in *Phospholipids: New Biochemistry* (Hawthorne, J. N., & Ansell, G. B., Eds.) Vol. 4, pp 359–434, Elsevier, Amsterdam.

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

Van Oort, M. G., Dijkman, R., Hille, J. D. R., & de Haas, G. H. (1985a) *Biochemistry 24*, 7987–7993.

Van Oort, M. G., Dijkman, R., Hille, J. D. R., & de Haas, G. H. (1985b) *Biochemistry 24*, 7993–7999.

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

Van Scharrenburg, G. J. M., Puijk, W. C., de Haas, G. H.,

& Slotboom, A. J. (1983) *Eur. J. Biochem. 133*, 83-89.
Van Scharrenburg, G. J. M., Puijk, W. C., Seeger, P. R., de Haas, G. H., & Slotboom, A. J. (1984a) *Biochemistry 23*, 1256-1263.
Van Scharrenburg, G. J. M., Jansen, E. H. J. M., Egmond, M. R., de Haas, G. H., & Slotboom, A. J. (1984b) *Biochemistry 23*, 6285-6294.
Van Wezel, F. M., & de Haas, G. H. (1975) *Biochim. Biophys. Acta 410*, 299-309.
Verger, R., & de Haas, G. H. (1973) *Chem. Phys. Lipids 10*, 127-136.
Verger, R., Mieras, M. C. E., & de Haas, G. H. (1973) *J. Biol. Chem. 248*, 4023-4034.
Volwerk, J. J., & de Haas, G. H. (1982) in *Molecular Biology of Lipid-Protein Interactions* (Griffith, O. H., & Jost, P., Eds.) pp 69-149, Wiley, New York.
Wallace, C. J. A., & Harris, D. E. (1984) *Biochem. J. 217*, 589-594.

Products of the Inactivation of Ribonucleoside Diphosphate Reductase from *Escherichia coli* with 2′-Azido-2′-deoxyuridine 5′-Diphosphate†

Scott P. Salowe, Mark A. Ator,‡ and JoAnne Stubbe*
Department of Biochemistry, College of Agriculture and Life Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53706

Received September 10, 1986; Revised Manuscript Received December 17, 1986

ABSTRACT: Ribonucleoside diphosphate reductase (RDPR) from *Escherichia coli* was completely inactivated by 1 equiv of the mechanism-based inhibitor 2′-azido-2′-deoxyuridine 5′-diphosphate (N_3UDP). Incubation of RDPR with [3′-^{3}H]N_3UDP resulted in 0.2 mol of ^{3}H released to solvent per mole of enzyme inactivated, indicating that cleavage of the 3′ carbon–hydrogen bond occurred in the reaction. Incubation of RDPR with [β-^{32}P]N_3UDP resulted in stoichiometric production of inorganic pyrophosphate. One equivalent of uracil was eliminated from N_3UDP, but no azide release was detected. Analysis of the reaction of RDPR with [$^{15}N_3$]N_3UDP by mass spectrometry revealed that the azide moiety was converted to 0.9 mol of nitrogen gas per mole of enzyme inactivated. The tyrosyl radical of the B2 subunit was destroyed during the inactivation by N_3UDP as reported previously [Sjöberg, B.-M., Gräslund, A., & Eckstein, F. (1983) *J. Biol. Chem. 258*, 8060-8067], while the specific activity of the B1 subunit was reduced by half. Incubation of [5′-^{3}H]N_3UDP with RDPR resulted in stoichiometric covalent radiolabeling of the enzyme. Separation of the enzyme's subunits by chromatofocusing revealed that the modification was specific for the B1 subunit.

Ribonucleoside diphosphate reductase (RDPR)[1] from *Escherichia coli* catalyzes the conversion of the four common ribonucleoside 5′-diphosphates to the corresponding 2′-deoxyribonucleotides. The enzyme is proposed to be a 1:1 complex of two subunits designated B1 and B2, each of which consists of two polypeptide chains (Thelander & Reichard, 1979; Sjöberg & Gräslund, 1983; Carlson et al., 1984). The B1 subunit ($\alpha\alpha'$, M_r 170 000) contains binding sites for the substrates and nucleoside triphosphate allosteric effectors which control the specificity and activity of the enzyme. Subunit B1 also contains redox-active thiols in each of its two active sites which become oxidized during turnover (Thelander, 1974). Reducing equivalents for the reaction are provided by NADPH through a transport chain consisting of the proteins thioredoxin and thioredoxin reductase, or glutaredoxin, glutathione, and glutathione reductase. The B2 subunit ($\beta\beta$, M_r 87 000) contains the cofactor that is unique to this enzyme, a binuclear iron center and organic free radical arising from the oxidation of a single tyrosine residue of the subunit. The tyrosyl radical has a characteristic visible absorbance at 410 nm and an EPR signal at $g = 2.0047$ that disappear when the radical is reduced by reagents such as hydroxyurea or hydroxylamine (Kjøller-Larsen et al., 1982). Although the tyrosyl radical is essential for enzyme activity, a specific role in catalysis has not been demonstrated.

We have proposed that the chemical mechanism of ribonucleotide reduction involves radical intermediates (Stubbe & Ackles, 1980; Ashley & Stubbe, 1986). The hypothetical radical mechanism has been supported by extensive studies with isotopically labeled substrates (Stubbe & Ackles, 1980; Stubbe et al., 1983) and the mechanism-based inhibitor 2′-chloro-2′-deoxyuridine 5′-diphosphate (ClUDP) (Stubbe & Kozarich, 1980; Harris et al., 1984; Ator & Stubbe, 1985). The first direct evidence for radical involvement in RDPR-mediated reactions, however, has come from studies with 2′-azido-2′-deoxycytidine 5′-diphosphate (N_3CDP) and 2′-

† This research was supported by U.S. Public Health Service Grant GM 29595 from the National Institutes of Health. J.S. is a recipient of an H. I. Romnes Fellowship from the University of Wisconsin and NIH Research Career Development Award AM 01222.

‡ Present address: Department of Pharmaceutical Chemistry, University of California, San Francisco, San Francisco, CA 94143.

[1] Abbreviations: RDPR, ribonucleoside diphosphate reductase; EPR, electron paramagnetic resonance; ClUDP, 2′-chloro-2′-deoxyuridine 5′-diphosphate; N_3CDP, 2′-azido-2′-deoxycytidine 5′-diphosphate; N_3UDP, 2′-azido-2′-deoxyuridine 5′-diphosphate; N_3ADP, 2′-azido-2′-deoxyadenosine 5′-diphosphate; NH_2UDP, 2′-amino-2′-deoxyuridine 5′-diphosphate; PP_i, inorganic pyrophosphate; P_i, inorganic phosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IP, ion pairing; HPLC, high-performance liquid chromatography; PEI, poly(ethylenimine); TLC, thin-layer chromatography; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; FDH, formate dehydrogenase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DEAE, diethylaminoethyl; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

0006-2960/87/0426-3408$01.50/0