Role of the N-Terminus in the Interaction of Pancreatic Phospholipase A₂ with Aggregated Substrates. Properties and Crystal Structure of Transaminated Phospholipase A₂[†]

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ABSTRACT: A free N-terminal α-NH$_3^+$ group is absolutely required for full catalytic activity of phospholipase A₂ on aggregated substrates. To elucidate how this α-NH$_3^+$ group triggers catalytic activity, we specifically transaminated this group in various pancreatic phospholipases A₂. Porcine, porcine iso-, equine, human, ovine, and bovine phospholipases A₂ all loose catalytic activity on micellar substrates due to the inability of the transaminated proteins to bind to neutral micellar substrate analogues, as was found for the zymogens. Loss of activity is pseudo first order, the rate constants being different for the enzymes studied. The transaminated phospholipases A₂ have an intact active site, as catalytic activities on monomeric substrates are comparable to those of the respective zymogens. The X-ray structure of transaminated bovine phospholipase A₂ at 2.1-Å resolution shows that the N-terminal region and the sequence 63–72 in this protein are more flexible than in the native enzyme. Also, in this respect, the transaminated enzyme very much resembles the zymogen structure. In good agreement with this, it was found by photochemically induced dynamic nuclear polarization ¹H NMR that aromatic resonances of Trp-3 and Tyr-69 are affected by transamination. In addition, fluorescence spectroscopy of the unique Trp-3 in transaminated bovine phospholipase A₂ revealed a red shift of the emission maximum indicative of a more polar environment of Trp-3 in the transaminated phospholipase A₂ as compared to the enzyme. The high mobility or disorder of the N-terminal region and of the 63–72 region is due to disruption of the hydrogen bonds of the α-NH$_3^+$ with the O$_{\epsilon 1}$ atom of Gln-4 and the carbonyl oxygen of Asn-71 by transamination. It is concluded that this increased mobility or disorder destroys affinity for aggregated phospholipids or, depending on the nature of the phospholipids, leads to unproductive binding.

Phospholipases A₂ (EC 3.1.1.4) are ubiquitous phospholipid-degrading enzymes that can be found both inside and outside the cell. A review on the occurrence and properties of the extracellular phospholipases A₂ has been published recently (Slotboom et al., 1982). These extracellular phospholipases are relatively small proteins (M_r 14 000 for the monomeric form) that require Ca²⁺ ions as a cofactor for hydrolysis. Moreover, their catalytic activity is strongly dependent upon the state of the substrate. Although monomeric substrates are hydrolyzed, the enzyme only becomes fully active in the presence of aggregated substrates. From the X-ray structure of bovine phospholipase A₂ (Dijkstra et al., 1981a,b) as well as from solution studies (Slotboom et al., 1982), there is independent evidence that the active site and the phospholipid binding site or IRS[1] are not only functionally but also topographically distinct sites.

It has been shown, both for the enzymes from mammalian pancreas (van Dam-Mieras et al., 1975; Slotboom & de Haas, 1975; Slotboom et al., 1977) and for several snake venom phospholipases (Verheij et al., 1981; J. van Eijk, personal communication) that the catalytic activity of these proteins for aggregated substrate is critically dependent on the presence

of a protonated α-amino group.

In order to further investigate the role of the N-terminus, we applied the transamination reaction (Dixon & Fields, 1972) successfully to mammalian pancreatic phospholipases A₂. As a result of this reaction the N-terminal residue Ala is converted into a pyruvoyl group and the enzyme is no longer active on aggregated substrates. The effect of transamination on the catalytic activity and substrate binding properties of phospholipase A₂ has been investigated. Interestingly, the modification appeared to have a dramatic effect on the interaction of phospholipase A₂ with aggregated substrates, but only a slight effect was observed on the activity toward monomeric substrates. The crystal structure of bovine transaminated phospholipase A₂, determined at 2.1-Å resolution, showed an unexpected partial disorder of the modified molecule in its N-terminal region and in the sequence 63–72. This disorder resembles closely that of prophospholipase A₂. These results permitted us to understand the role of the N-terminus in the interaction with aggregated substrates.

Experimental Procedures

Materials

Porcine, bovine, ovine, and equine pancreatic prophospholipases A₂ were purified and converted into their corresponding phospholipases A₂ as described previously (Nieuwenhuizen et al., 1974; Dutihl et al., 1975; Evenberg et al.,

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[1] Abbreviations: DEAE, diethylaminoethyl; CM, carboxymethyl; MPD, 2-methyl-2,4-pentanediol; CIDNP, chemically induced dynamic nuclear polarization; NMR, nuclear magnetic resonance; MHz, megahertz; rms, root mean square; IRS, interface recognition site; Tris, tris(hydroxymethyl)aminomethane; F_o, observed structure factor; F_c, calculated structure factor; TSP, sodium trimethylsilyl[2,2,3,3-²H₄]-propionate; ppm, parts per million; $R = \sum_{hkl} ||F_o| - |F_c||/\sum_{hkl}|F_o|$.

1977). Porcine pancreatic isophospholipase A_2 was obtained as described by van Wezel & de Haas (1975). Human phospholipase A_2 was a gift of Dr. H. M. Verheij.

DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were purchased from Whatman (Kent, England) and various Sephadex products from Pharmacia Fine Chemicals (Uppsala, Sweden) and prepared for use according to the manufacturer's recommendations. Sodium glyoxylate monohydrate was purchased from Fluka AG (Buchs, Switzerland). The sodium glyoxylate was recrystallized twice as described by Radin & Metzler (1955). n-Hexadecyl- and n-octadecylphosphocholines were prepared as described previously (van Dam-Mieras et al., 1975). 1,2-Dioctanoyl-sn-glycero-3-phosphocholine was prepared as described by Cubero Robles & van den Berg (1969). 2,3-Bis(hexanoylthio)propylphosphocholine was synthesized as described by Volwerk et al. (1979). All other chemicals were of the highest purity available.

Methods

Amino acid analyses were performed by the method of Spackman et al. (1958) on a Beckman Unichrom amino acid analyzer, equipped with a high-sensitivity attachment, or on a Technicon TSM amino acid analyzer. Samples were hydrolyzed for 24 h at 110 °C in evacuated, sealed tubes with 5.8 N HCl. Tryptophan was determined similarly by addition of 4% thioglycolic acid as described by Matsubara & Sasaki (1969).

Protein concentrations were determined from the absorbancies at 280 nm using an $E_{1cm}(1\%)$ value of 13.0 for porcine and bovine phospholipases A_2, whereas values of 12.3 and 13.6 were used for horse and human phospholipases A_2, respectively.

Enzyme activities of all pancreatic phospholipases A_2 were determined routinely by using the titrimetric assay procedure with egg-yolk lipoproteins as substrates (Nieuwenhuizen et al., 1974). In contrast to the described procedure a 2-fold higher Ca^{2+} concentration and an 1.5-fold higher sodium deoxycholate concentration were used for the various phospholipases except for the human enzyme. The enzymatic activity of the human phospholipase A_2 was assayed similarly with 10 mL of an egg-yolk suspension (one egg yolk suspended in 100 mL of 5 mM $CaCl_2$ solution) and 20 mL of a sodium deoxycholate solution (3.74 g/L). The fatty acids released were titrated with standardized 0.02 N NaOH. Kinetic measurements using micellar 1,2-dioctanoyl-sn-glycero-3-phosphocholine and monomeric 2,3-bis(hexanoylthio)propyl-phosphocholine were performed as described before (de Haas et al., 1971; Volwerk et al., 1979, respectively).

Slab gel electrophoresis was performed for 1 h at 50 mA on 1.5 mm thick 7.5% polyacrylamide gels at pH 4.3 and 9.5 in an apparatus as described by Studier (1973). The gels and buffer solutions were prepared according to the manufacturer's instructions (Shandon, England). Protein staining was done with a 0.1% solution of amido black in 7% acetic acid for 1 h and destaining in 7% acetic acid overnight.

N-Terminal amino acid residues were determined by dansylation according to the method of Gray (1972).

NMR Spectroscopy. ^{1}H NMR (360-MHz) spectra were obtained with a Bruker HX-360 spectrometer at the SON Facility in Groningen, The Netherlands, using quadrature detection in the pulsed Fourier transform mode. Photo-CIDNP spectra (Kaptein, 1978) were recorded as previously described (Jansen et al., 1980; Egmond et al., 1980) with N-3-carboxymethyllumiflavin as the dye. Dioxane was added as internal standard. However, chemical shifts were calculated relative to TSP by using 3.747 for the chemical shift difference between dioxane and TSP.

Fluorescence Measurements. Fluorescence spectra were recorded at 25 °C with a Perkin-Elmer MPF-3 spectrofluorometer using 1-cm cells as described previously (van Dam-Mieras et al., 1975).

Ultraviolet Difference Spectroscopy. Ultraviolet difference spectra were obtained at 25 °C with an Aminco DW-2A spectrophotometer equipped with an automatic base line correction accessory (Midan data analyzer from Aminco) as previously described (van Dam-Mieras et al., 1975; Hille et al., 1981).

Transamination of pancreatic phospholipases A_2 (1 mg/mL) was performed in 2 M sodium acetate–0.4 M acetic acid at pH 5.5 and 25 °C with sodium glyoxylate monohydrate (0.1 M) in the presence of 10 mM $Cu(NO_3)_2$ as described by Dixon & Fields (1972). On a preparative scale, transamination of porcine and bovine phospholipases A_2 was done similarly but now in the presence of 6 M guanidine hydrochloride. The reaction was stopped when >90% inactivation was reached (30–40 min) by gel filtration on a Sephadex G-25 coarse column (120 × 1.5 cm) equilibrated with 1% $(NH_4)HCO_3$ at 4 °C. The protein fraction was dialyzed for 24 h, and the transaminated protein was purified by ion-exchange chromatography on CM-cellulose and DEAE-cellulose using conditions as described for the native enzymes (Nieuwenhuizen et al., 1974; Dutihl et al., 1975). Extensive dialysis (24 h) at basic pH was done after each column to remove salt and noncovalently bound glyoxylate.

Crystallization and Data Collection. Crystallization of transaminated bovine phospholipase A_2 was accomplished as described previously for the native enzyme from 50% MPD in Tris-HCl buffer, pH 7.6 (Dijkstra et al., 1978). The space group is $P2_12_12_1$ with cell dimensions $a = 46.68$ Å, $b = 65.01$ Å, and $c = 38.04$ Å, i.e., differing less than 1% from those of the native enzyme. Intensity data were measured from four crystals on an Enraf-Nonius CAD4F diffractometer to a resolution of 2.1 Å. These data were processed as described previously (Dijkstra et al., 1981a). Altogether 5435 reflections out of the possible 7160 to 2.1 Å were included in the final data set (76%). The overall agreement factor

$$R_{agree} = \sum_{hkl} \sum_i |F_i - \bar{F}| / \sum_{hkl} \bar{F}$$

for multiply measured reflections was 6.2%.

Crystallographic Refinement. Because the cell dimensions of native and transaminated phospholipase A_2 were so close, we took the refined structure of native phospholipase A_2 as the starting model for the refinement (Dijkstra et al., 1981a). No solvent molecules were included in the model. The crystallographic refinement was performed according to the restrained least-squares procedure of Konnert & Hendrickson (1980). The data pertinent to the weighting scheme used in the refinement can be found in the supplementary material. After each series of refinement cycles a $(F_{obsd} - F_{calcd})$ or a $(2F_{obsd} - F_{calcd})$ Fourier was calculated, and the model together with this difference map was examined on an Evans and Sutherland picture system II using the program GUIDE (Brandenburg et al., 1981). When necessary the model was rebuilt. The coordinates of the transaminated bovine phospholipase A_2 have been deposited with the Brookhaven/Cambridge Protein Data Bank.

Results

Transamination of Various Pancreatic Phospholipases. Reaction of sodium glyoxylate monohydrate with various pancreatic phospholipases A_2 resulted in the loss of enzymatic activity when assayed in the egg-yolk assay. The loss of

Table I: Half-Time Values for Loss of Catalytic Activity (Egg-Yolk Assay) of Pancreatic Phospholipases A$_2$ by Reaction with Sodium Glyoxylate Monohydrate[a]

enzyme	addition	$t_{1/2}$ (min)
porcine phospholipase A$_2$		53
	n-hexadecyl-PC[b] (35 mM)	∞
	Gdn·HCl (6 M)	5
equine phospholipase A$_2$		16
	Gdn·HCl (6 M)	5
bovine phospholipase A$_2$		400
	Gdn·HCl (6 M)	5
porcine isophospholipase		900
human phospholipase A$_2$		13
ovine phospholipase A$_2$		700

[a] Conditions: 25 °C; 2 M sodium acetate, 0.4 M acetic acid, 10 mM Cu^{2+}, pH 5.5; protein concentration 71.4 μM; 0.1 M sodium glyoxylate monohydrate. [b] PC is phosphocholine; Gdn·HCl is guanidine hydrochloride.

catalytic activity with time was found to be a pseudo-first-order process. The half-time values ($t_{1/2}$) for the inactivation, calculated from semilog plots of the percentage activity remaining vs. time are shown in Table I. Straight lines were observed through 90% inactivation. As can be seen from Table I equine, porcine, and human phospholipases A$_2$ are rather rapidly inactivated; half-time values of 16, 53, and 13 min, respectively, were found. In contrast, bovine and sheep phospholipases A$_2$ as well as porcine isophospholipase A$_2$ are only very slowly inactivated with half-time values of 400, 700, and 900 min, respectively. Due to unfolding of the proteins by 6 M guanidine hydrochloride, the rate of inactivation is greatly enhanced and the $t_{1/2}$ values for bovine, porcine, and equine phospholipases A$_2$ become equal to 5 min. The presence of 6 M guanidine hydrochloride was found not to be responsible for the loss of catalytic activity because full catalytic activity was recovered after removal of guanidine hydrochloride from phospholipase A$_2$ solutions in the absence of sodium glyoxylate. Due to precipitation of calcium glyoxylate, the effect of Ca^{2+} on the inactivation rate of phospholipase A$_2$ could not be determined. Similarly, the effect of monomeric substrate analogues could not be assessed because of the high salt concentration of the transamination medium, which dramatically decreases the critical micelle concentration. Micelles of the substrate analogue n-hexadecylphosphocholine almost absolutely protect against inactivation of porcine pancreatic phospholipase A$_2$ by transamination (Table I).

Properties of Transaminated Bovine and Porcine Phospholipases A$_2$. After purification as described under Methods the transaminated bovine and porcine phospholipases A$_2$ were found to be homogeneous on disc gel electrophoresis at pH 9.4 and 4.5. Dansylation of the transaminated phospholipases A$_2$ did not show dansyl-Ala, indicating the absence of the α-NH$_2$ group of the N-terminal Ala residue. In good agreement, the amino acid composition of the transaminated porcine pancreatic phospholipase A$_2$ (including Trp) was found to be identical with that of the native enzyme, except for the loss of one Ala residue.[2] These transaminated phospholipases A$_2$ were found to be devoid of catalytic activity both in the egg-yolk assay (pH 8) and in the micellar 1,2-dioctanoyl-lecithin assay (pH 6). When assayed on the monomeric substrate DL-[2,3-bis(hexanoylthio)propyl]phosphocholine, however, the transaminated phospholipases A$_2$ were found to

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[2] The amino acid analyses of native and transaminated porcine pancreatic phospholipases A$_2$ were submitted to the reviewers for examination and are given in the supplementary material (see paragraph at end of paper regarding supplementary material).
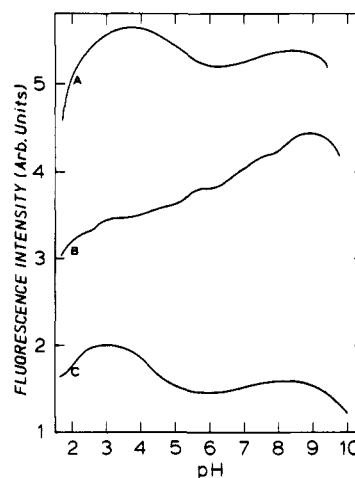

FIGURE 1: pH dependence of fluorescence intensities of bovine prophospholipase A$_2$ (A), bovine phospholipase A$_2$ (B), and transaminated bovine phospholipase A$_2$ (C). Experimental conditions: 1 mM Tris, 1 mM sodium acetate, 0.1 M NaCl, 14.2 μM protein; excitation 295 nm; 25 °C. Intensities were measured at the emission maxima (350 nm, curve A; 343 nm, curve B; 352 nm, curve C).

be catalytically active. For the transaminated porcine phospholipase A$_2$, a V_{max} value of 10 ± 1.5 μequiv min^{-1} mg^{-1} was found, as compared to 2 ± 0.2 and 14 ± 3 μequiv min^{-1} mg^{-1} for the native porcine phospholipase A$_2$ and prophospholipase A$_2$, respectively, reported previously by Volwerk et al. (1979). The observed K_m values for these three proteins were found to be 1 mM.

As the modified phospholipases have lost their activity toward micellar substrate but not toward monomeric substrate, we determined the influence of transamination on substrate binding properties of the porcine phospholipase A$_2$ to micelles of substrate analogues. Binding of phospholipid micelles to pancreatic phospholipases A$_2$ can easily be measured by fluorescence and ultraviolet difference spectroscopy. Upon binding, the fluorescence intensity of the unique Trp-3 residue in the lipid binding domain increases and simultaneously there is a shift in the emission maximum from 243 to 232 nm (van Dam-Mieras et al., 1975). With UV difference spectroscopy the binding results in difference spectra characteristic for Trp and Tyr perturbations (Hille et al., 1981). When fluorescence spectroscopy was studied at pH 6 in the absence and presence of 0.1 M Ca^{2+}, no increase in fluorescence intensity nor a blue shift of the emission maximum was observed upon addition of increasing amounts of n-octadecylphosphocholine to transaminated porcine phospholipase A$_2$, in contrast to the native enzyme. Similarly, no binding of transaminated porcine phospholipase A$_2$ to micellar n-octadecylphosphocholine could be detected at pH 6 in the presence of 0.1 M Ca^{2+} by ultraviolet difference spectroscopy. Apparently, the lack of catalytic activity of transaminated phospholipase A$_2$ toward micellar substrates is due to the loss of affinity for neutral lipid–water interfaces.

Fluorescence Spectroscopy. As shown previously (van Dam-Mieras et al., 1975) the unique Trp-3 is an attractive reporter group for conformational changes in the N-terminal region of pancreatic phospholipase A$_2$. We therefore investigated the pH dependence of the fluorescence intensity of transaminated bovine phospholipase A$_2$ in comparison with that of the native enzyme and its zymogen (Figure 1). The titration curve found for the transaminated bovine phospholipase A$_2$ (C, Figure 1) differs considerably from that of the native enzyme (B, Figure 1) but resembles much more that of the zymogen (A, Figure 1) lacking also the free α-NH$_3^+$
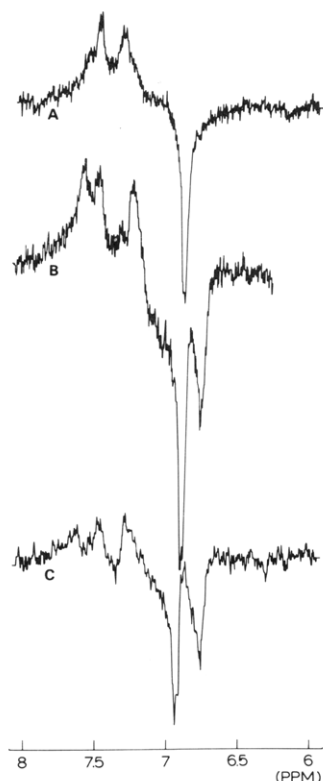
FIGURE 2: Aromatic region of photo-CIDNP ^1H NMR spectra at 360 MHz for native porcine phospholipase A_2 (A), prophospholipase A_2 (B), and transaminated phospholipase A_2 (C). Spectra were recorded for 2 mM protein solutions at 40 °C, pH 5, in the presence of 0.2 mM flavin dye.

Laser Photo-CIDNP ^1H NMR. The effect of transamination was also studied by laser photo-CIDNP ^1H NMR (Egmond et al., 1980). Figure 2 shows the CIDNP difference spectra of porcine phospholipase A_2 (A), its zymogen (B), and the transaminated enzyme (C). Absorptive signals (upward) at approximately 7.3–7.7 ppm stem from Trp-3 aromatic resonances. Especially for the transaminated enzyme these resonances are rather weak. Downward (emissive) signals arise from aromatic protons of Tyr residues. The strong signal at 6.9 ppm is assigned to $H_{3,5}$ protons of Tyr-123. The weaker signal at approximately 6.7 ppm, observed in traces B and C of Figure 2, stems from $H_{3,5}$ protons of Tyr-69. The CIDNP signal of Tyr-69 $H_{3,5}$ protons in the porcine enzyme overlaps with the Tyr-123 $H_{3,5}$ signal under the conditions used. For the assignment of the above-mentioned resonances, the reader is referred to Jansen et al. (1978) and Egmond et al. (1980).

Crystallographic Refinement. The crystallographic refinement of transaminated bovine phospholipase A_2 with native phospholipase A_2 as the starting model proceeded without major difficulties, and convergence was rapid. A summary of the refinement, the weights used, and the deviation from ideal geometry of the starting and final models is given in the supplementary material. From a difference Fourier calculated after the first five refinement cycles it appeared that no density was available for Ala-1, Leu-2, the side chain of Trp-3, and the loop from residue 63–72, i.e., in total about 10% of the protein molecule. Consequently, these residues were left out from the model in all subsequent refinement cycles. Even at the end of the refinement no density was present for these residues.

During the first five refinement cycles an overall temperature factor of 15 $Å^2$ was used, and only the positional parameters were refined. From cycle 6 onward individual temperature factors as present in the model of native bovine phospholipase were given to the atoms, and we refined both positional parameters and temperature factors.

At the conclusion of the refinement, after 39 refinement cycles, the final crystallographic R factor is 0.173 for all data between 2.1 and 7.1 Å. The final electron density maps were of excellent quality: e.g., in a $(2F_{obsd} - F_{calcd})$ difference Fourier map, many carbonyl oxygen atoms showed up clearly, and it can be concluded that the final model has a fairly acceptable geometry. In accordance with other structures refined at a similar resolution, we estimate the rms error in the coordinates to be about 0.15–0.20 Å for the well-defined atoms [see, e.g., Stenkamp et al. (1982)]. The R factor of 0.173 indicates a good correspondence between the final model and the observed structure factors. Apparently the residues that were left out
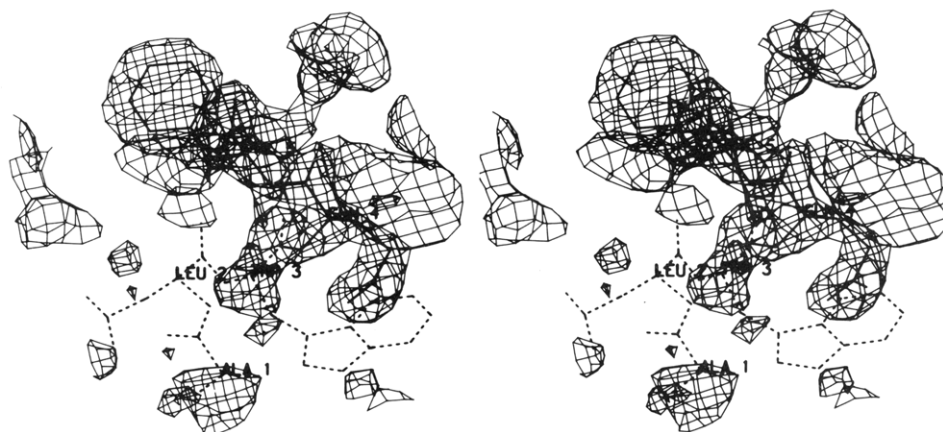
group of Ala-1. Deprotonation of the α-NH_3^+ group gives rise to an increase in fluorescence intensity above pH 8 in the bovine enzyme, but not in the transaminated phospholipase A_2. Similarly, the emission maximum of the transaminated enzyme (352 nm) resembles much more that of the zymogen (350 nm) than that of the native enzyme (343 nm). The high emission maxima of the former two proteins indicate that the Trp-3 side chain in both proteins is in a more polar environment as compared with that of the native enzyme. The fluorescence intensity of transaminated phospholipase A_2, however, is much lower as compared to that of the native enzyme as well as of the zymogen. Apparently Trp-3 in the transaminated enzyme is in a more quenching environment differing from that in the zymogen, despite the similarity in emission maxima and shape of the titration curves of both proteins.

FIGURE 3: Stereo picture of the electron density of transaminated phospholipase A_2 after completion of the crystallographic refinement. The broken lines indicate that no reliable positions could be obtained for residues 1 and 2 and the side chain of Trp-3.
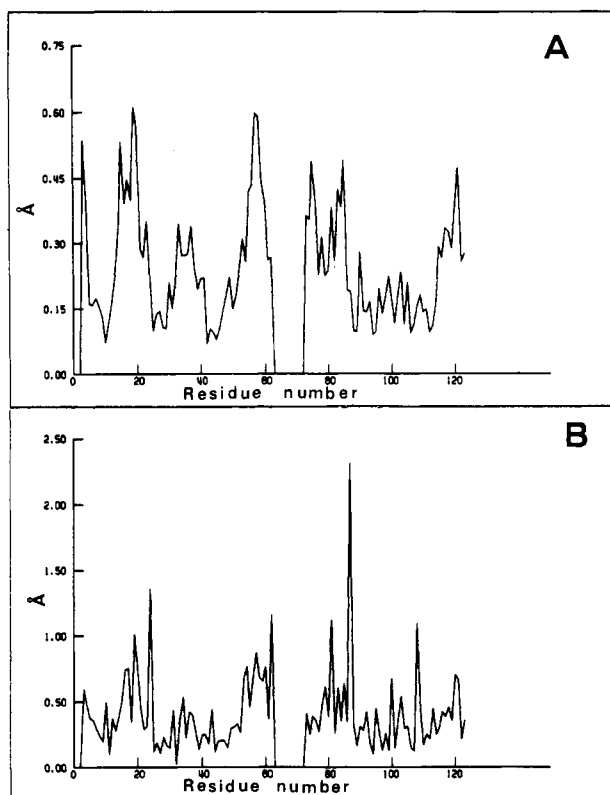
FIGURE 4: Root mean square deviations for the N, C$_\alpha$, and C atoms (A) and for the side-chain atoms (B) after optimal superposition of the atomic positions of phospholipase A$_2$ (Dijkstra et al., 1981a) and transaminated phospholipase A$_2$.

from the model do not contribute to F_{obsd} to a large extent. This implies that these residues are disordered in the crystals or have a high mobility. Figure 3 shows the sudden disappearance of the electron density at Trp-3. A total of 86 water molecules was included in the final model, but, in contrast to the native bovine phospholipase A$_2$ model (Dijkstra et al., 1981a), no MPD was included. In the final difference map we could not obtain unambiguous evidence for the presence of MPD in the crystal structure.

The overall rms shift of the main-chain N, C$_\alpha$, and C atoms between the starting and final model is 0.60 Å. This rms shift is, however, mainly caused by a slight rotation (2.0°) and translation (0.67 Å) of the transaminated phospholipase molecule in the crystal cell with respect to the native enzyme. After optimal superimposition of the native and transaminated enzyme models, allowing for this rotation and translation, the rms difference between the respective main-chain N, C$_\alpha$, and C atoms is 0.28 Å for 111 residues.

Comparison of Crystal Structures of Native and Transaminated Bovine Phospholipase A$_2$. The most conspicuous difference in the three-dimensional structures of native and transaminated bovine phospholipase A$_2$ is the disorder or high mobility of about 10% of the structure of the transaminated enzyme with respect to the native enzyme. These disordered regions are residues 1 and 2, the side chain of residue 3, and the loop from residue 63 to residue 72. The remaining 90% of the structure is very similar: the rms difference between all equivalent protein atoms (main chain and side chain), which are visible in the electron density maps, is 0.35 Å. As mentioned above, the rms difference between the 111 equivalent main-chain N, C$_\alpha$, and C atoms is 0.28 Å. This is within the sum of the estimated errors in the two structures. Only four residues deviate by more than 0.56 Å in their main-chain atoms (Figure 4A), and these residues are in regions with relatively high temperature factors: Leu-19, Leu-20, Lys-57, and Leu-58. Also, most of the side chains have a similar position in both molecules, although the positional differences are larger than for the main chain atoms (Figure 4B). The Glu-87 side chain has a different orientation in the two molecules. However, because the Glu-87 is at the surface of the molecule and points into the solvent, this conformational difference is not considered significant. The side chains of the residues constituting the active site (Dijkstra et al., 1981a) are very similar indeed. Their rms positional difference is 0.20 Å. Also, the water molecule in the active site proposed to play an essential role in the catalytic process occupies a virtually identical position.

Discussion

The transamination reaction allows the selective modification of the α-amino group of proteins and so permits the function of this group to be studied specifically. This minor modification is specific for the α-NH$_3^+$ group of the N-terminal residue, which is transformed into an oxygen atom. In general, it has been found that the reaction proceeds rapidly, being essentially complete within $^1/_2$–1 h (Dixon & Fields, 1972). However, in the case of the pancreatic phospholipases (Table I), as well as with the snake venom phospholipases (Verheij et al., 1981), the modification reaction turned out to be much slower, with half-time values of up to 15 h. Only after the unfolding of the peptide chain by treatment with 6 M guanidine hydrochloride or 4 M N,N,N',N'-tetramethylurea could a rate of inactivation be attained, which was comparable to those given in other reports (Dixon & Fields, 1972). Most probably the low susceptibility of phospholipase's N-terminus for the transamination reagent is caused by the restricted accessibility of the α-NH$_3^+$ group. From the crystal structures
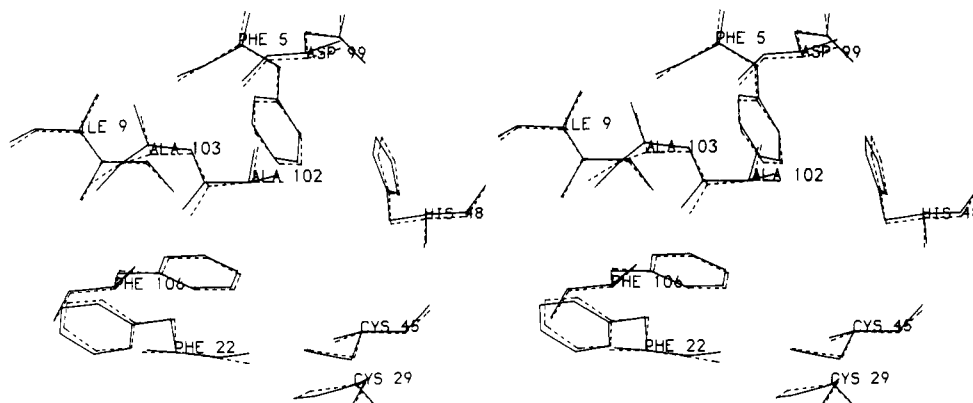

FIGURE 5: Stereo drawing with a comparison of the active sites in the native (broken lines) and transaminated phospholipase A$_2$.
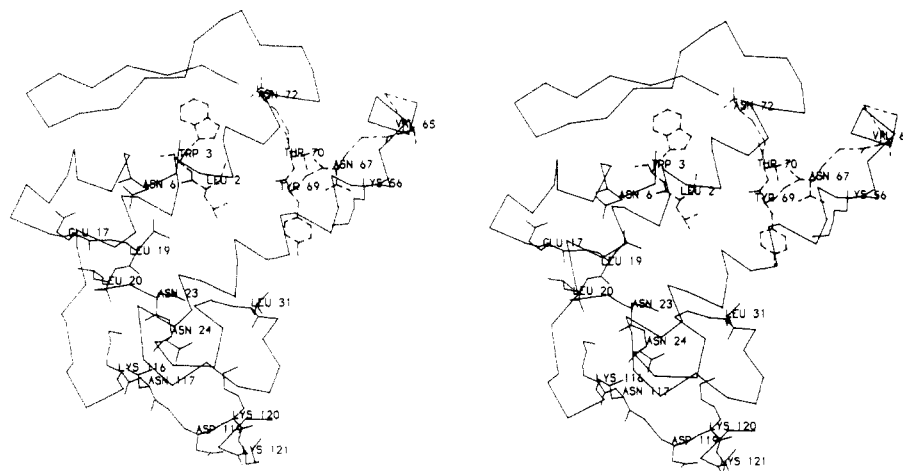
FIGURE 6: Stereo diagram showing the residues proposed to be involved in the binding of aggregated phospholipids (Dijkstra et al., 1981b). Residues found to be mobile in the crystal structure of transaminated bovine phospholipases are represented by broken lines.

of bovine (Dijkstra et al., 1981a) and porcine phospholipases A_2 (Dijkstra et al., 1983), it appeared that the N-terminus is hidden in the protein's interior, making hydrogen bonds with the $O_{\epsilon 1}$ atom of Gln-4 and the carbonyl oxygen of residue 71. A third hydrogen bond is formed with an internal water molecule. Previously it was found from the X-ray structures of porcine and bovine phospholipases A_2 (Dijkstra et al., 1983) and from the pK_a values of the α-amino groups of these enzymes, 8.4 and 8.9, respectively (Slotboom et al., 1978; Jansen et al., 1979), that in the porcine enzyme the N-terminus is somewhat more open to the solvent than in the bovine enzyme. These findings may very well explain the differences in reactivity between these two enzymes, although we cannot exclude an effect of the local charge distribution around the N-terminus on the rate of the reaction. In this respect, it has to be mentioned that similar substitutions of the near-invariant Gln-4 in porcine and bovine phospholipases A_2 also lead to different effects on catalytic activity and substrate binding properties (van Scharrenburg et al., 1984). As a result of the transamination reaction the modified enzyme has lost its catalytic activity toward aggregated substrates, but still has retained its activity on monomeric substrates. Both the K_M and V_{max} parameters for the degradation of monomeric substrates are, within the estimated error range, between the values found for phospholipase A_2 and prophospholipase A_2. This suggests that the active sites in these enzymes are identical (Dijkstra et al., 1982). As inferred from the crystal structure of bovine phospholipase, the active site is made up of several invariant residues: His-48, Asp-99, Phe-5, Ile-9, Phe-22, Ala-102, Ala-103, Phe-106, and the disulfide bridge between Cys-29 and Cys-45 (Dijkstra et al., 1981b). Also the calcium ion, which is essential for catalytic activity, is in this region. From a comparison of the crystal structures of native and transaminated bovine phospholipase A_2 it is obvious that no conformational change in the active site has occurred as a result of the modification (Figure 5). Also the calcium ion and the putative catalytic water molecule occupy identical positions in the two molecules. Thus, the active sites of native and transaminated phospholipase A_2 are identical.

In contrast, the catalytic activity of the transaminated enzyme toward neutral micellar substrates has completely disappeared. As shown from the UV and fluorescence difference spectroscopy studies, this loss of activity can be attributed to the fact that the modified pancreatic enzyme does no longer bind to micellar substrates. In order to elucidate the molecular basis of this loss of affinity for aggregated substrates, we

compared the crystal structures of native and transaminated bovine phospholipase. The most conspicuous difference is the high mobility or disorder in about 10% of the molecule, comprising residues 1–3 and 63–72.

From solution studies a higher mobility in these parts of the molecule can be inferred. This was previously shown for Tyr-69 in bovine prophospholipase A_2, which is more mobile than Tyr-69 in the bovine phospholipase A_2 as observed by photo-CIDNP ^{1}H NMR (Egmond et al., 1983). The mobile Tyr-69 residue gives rise to aromatic $H_{3,5}$ proton resonances approximately 0.2 ppm downfield from their position in the native enzyme. This downfield shift was also noted for transaminated bovine and porcine phospholipases A_2 and porcine prophospholipase A_2. These latter proteins do not show any enhanced activity on aggregated substrates. Also, for native porcine phospholipase A_2 the Tyr-69 $H_{3,5}$ CIDNP ^{1}H NMR signal was found to shift downfield by approximately 0.2 ppm when the pH was increased to 9 in the absence of calcium ions (data not shown). Under these conditions the α-amino group becomes deprotonated and the enzyme, resembling the zymogen, is no longer able to bind to micellar substrate analogues (Donné-Op den Kelder et al., 1981). The data were found to be less clear for Trp-3 resonances. Bovine phospholipase A_2 and proenzyme contain fully exposed and mobile Trp-3 residues (Egmond et al., 1980, 1983), while porcine enzymes probably contain much less mobile Trp-3 residues as derived from the weak and poorly resolved CIDNP ^{1}H NMR signals. On the basis of the fluorescence data and the chemical shifts noted for Trp-3 aromatic resonances in bovine enzymes, the transaminated phospholipase A_2 and the proenzyme contain Trp-3 residues present in a more polar environment than in the active enzyme. Probably a transition from a rigid helical to a more flexible disordered or random-coil conformation of the N-terminal region has occurred in the transaminated enzyme, comparable with the N-terminal region of the proenzyme. This result is fully in agreement with the crystallographic data.

In the crystal structure these mobile residues comprise about one-third of the proposed binding site (IRS) for aggregated phospholipids (Dijkstra et al., 1981b). In the bovine enzyme this binding site consists of the following residues: *Leu-2*, *Trp-3*, Asn-6, Glu-17, Leu-19, Leu-20, Asn-23, Asn-24, Leu-31, Lys-56, *Val-65*, *Asn-67*, *Tyr-69*, *Thr-70*, *Asn-72*, Lys-116, Asn-117, Asp-119, Lys-120, and Lys-121. The residues in italics acquire a high mobility in the transaminated enzyme (see also Figure 6). On the basis of kinetic and amino

acid modification studies, several of these residues have been demonstrated to be involved in lipid binding [see for a review Slotboom et al. (1982)]. The high mobility of part of this lipid binding site causes the ΔG of binding to aggregated substrates to be less favorable than in the native enzyme. Thus, in the transaminated phospholipase the binding will be impaired, or even, it may result in no measurable binding at all, as we have found with the transaminated pancreatic phospholipases. With the snake venom enzymes (Verheij et al., 1981) binding of micellar substrates could still be observed, albeit with reduced affinity. In the latter case, despite the binding to interfaces, no hydrolysis of the aggregated substrate took place, even if the enzyme was saturated with substrate. The most simple explanation for this is that the active site of the modified enzyme is not properly oriented toward the interface. This is easily conceivable, because in the transaminated enzyme that part of the lipid binding site, which has not become mobile, is asymmetrically located around the active site (see Figure 6). Another explanation might be that for activity on micelles a special conformation of the phospholipase molecule is required, which is not possible in the transaminated enzyme. So far no evidence whatsoever has been obtained to corroborate this latter possibility.

The minute chemical modification described in this paper shows that substitution of the α-NH$_3^+$ group by another, small moiety causes an essential part of the lipid binding site of phospholipase A$_2$ to become mobile. Interestingly, virtually the same residues as in the transaminated enzyme were found to be highly disordered or mobile in the crystal structure of bovine prophospholipase A$_2$ (Dijkstra et al., 1982). This proenzyme is seven residues longer at the N-terminus, and its properties resemble those of the transaminated enzyme in many respects: it is active on monomeric substrates, but it is not able to degrade aggregated substrates; it has no free α-NH$_3^+$ group at Ala-1, and a substantial part of the lipid binding site has been loosened up.

Several other modifications affecting the α-NH$_3^+$ of the Ala-1 group have been reported: replacement of L-Ala by D-Ala (Slotboom et al., 1977) also destroys the activity of phospholipase A$_2$ with respect to neutral aggregated substrates.

Thus, as a conclusion, the function of the α-NH$_3^+$ group is to lock part of the lipid binding site (IRS) into a conformation proper to bind to phospholipid aggregates. This is effected by a hydrogen bond between the α-NH$_3^+$ group and the carbonyl oxygen of residue 71 (Dijkstra et al., 1983). Any modification that affects this hydrogen bond results in an enzyme in which part of the lipid binding site has become mobile or disordered. This causes an impaired affinity for aggregated phospholipids, or, depending on the exact nature of the phospholipids used, unproductive binding, resulting in an enzyme that has no activity toward aggregated substrates.

Acknowledgments

Supplementary Material Available

Tables containing input parameters and results of the restrained least-squares refinement and amino acid composition of native and transaminated porcine pancreatic phospholipase A$_2$ (2 pages). Ordering information is given on any current masthead page.

Registry No. DL-[2,3-Bis(hexanoylthio)propyl]phosphocholine, 70504-26-6.

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Inhibition of the Elastase of *Pseudomonas aeruginosa* by N^α-Phosphoryl Dipeptides and Kinetics of Spontaneous Hydrolysis of the Inhibitors[†]

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ABSTRACT: The rates of hydrolysis of N-[(α-L-rhamnopyranosyloxy)phospho]-L-leucyl-L-tryptophan (phosphoramidon), N^α-phosphoryl-L-leucyl-L-tryptophan (PO$_3$LeuTrp), N^α-phosphoryl-L-leucyl-L-phenylalanine (PO$_3$LeuPhe), and N^α-phosphoryl-L-leucyl-L-phenylalaninamide (PO$_3$LeuPheNH$_2$) were followed by proton nuclear magnetic resonance spectroscopy. The rates of hydrolysis (k_{obsd}) of PO$_3$LeuTrp, PO$_3$LeuPhe, and PO$_3$LeuPheNH$_2$ were all first order in phosphorylamide concentration over the pH range studied (3.8–9.5). The values for k_{obsd} at pH 7.3 and 37 °C are as follows: PO$_3$LeuTrp, 0.35 h^{-1}; PO$_3$LeuPhe, 0.63 h^{-1}; PO$_3$LeuPheNH$_2$, 0.73 h^{-1}. The values for k_{obsd} do not significantly change between pH 5 and pH 8 but dramatically decreased with increasing pH. The hydrolysis of PO$_3$LeuPhe and PO$_3$LeuPheNH$_2$ above a pH of approximately 5 was positively correlated with the concentration of monoanionic species (NHRPO$_3$H)$^{1-}$, and the values for the first-order rate constants for the respective monoanionic species were calculated to be 0.66 ± 0.03 h^{-1} and 1.07 ± 0.10 h^{-1}. Phosphoramidon was not found to hydrolyze after 6 days at 37 °C at a pH of 4.6 and 7.7, while the phosphorylamide PO$_3$LeuTrp, synthesized by the removal of L-rhamnose from phosphoramidon by base hydrolysis, was found to rapidly hydrolyze under these conditions. Solvolysis in aqueous methanol of PO$_3$LeuPhe and PO$_3$LeuPheNH$_2$ indicates that the hydrolysis reaction is bimolecular, proceeding by way of direct attack of solvent (H$_2$O, CH$_3$OH) on phosphorus. The proteolytic activity of elastase from *Pseudomonas aeruginosa* was measured with both hide powder azure and furylacryloyl-L-alanyl-L-phenylalaninamide in the presence and absence of PO$_3$LeuPhe, PO$_3$LeuPheNH$_2$, phosphoramidon, and PO$_3$LeuTrp. The relative degree of inhibition observed with both of these substrates was PO$_3$LeuTrp > PO$_3$LeuPhe ∼ phosphoramidon > PO$_3$LeuPheNH$_2$.

Several natural and synthetic low molecular weight reversible inhibitors of metalloproteinases are known. N-[(α-L-Rhamnopyranosyloxy)phospho]-L-leucyl-L-tryptophan, which was isolated from the culture filtrates of an actinomycete, *Streptomyces tanashiensis* (Suda et al., 1973), inhibits several metalloproteinases, including pseudomonas elastase (Komiyama et al., 1975; Umezawa et al., 1972), thermolysin (Morihara & Tsuzuki, 1980; Nishino & Powers, 1980; Kessler et al., 1982), and a membrane-bound peptidase from porcine kidney (Mumford et al., 1981). N-[[(6-Deoxy-α-L-talopyranosyl)oxy]phospho]-L-leucyl-L-tryptophan, which was isolated from *Streptomyces mozunensis* MK-23 (Murao et al., 1980), inhibits thermolysin (Kitagishi & Hiromi, 1983; Kitagishi et al., 1983). Synthetic phosphorus-containing metalloproteinase inhibitors include N-[(α-L-rhamnopyranosyl-

oxy)phospho] peptides (Komiyama et al., 1975), phosphoryl peptides[1] (Galardy, 1980; Holmquist & Vallee, 1979; Kam et al., 1979; Kessler et al., 1982; Murao et al., 1982a,b; Nishino & Powers, 1979), mono- and diesterified phosphoryl peptides (Galardy, 1982; Holmquist, 1977; Holmquist & Vallee, 1979; Thorsett et al., 1982), thiophosphoryl peptide (Nishino & Powers, 1979), and phosphonic acids and amides (Galardy et al., 1983). Phosphoryl peptides are potent reversible inhibitors

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