Comparative Studies of Tyrosine Modification in Pancreatic Phospholipases. 1. Reaction of Tetranitromethane with Pig, Horse, and Ox Phospholipases A_2 and Their Zymogens†

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ABSTRACT: Reaction of horse, pig, and ox pancreatic phospholipases A_2 and their zymogens with a 10-fold molar excess of tetranitromethane at pH 8 resulted in a considerable loss of enzymatic activity. In the presence of egg yolk lysolecithin micelles and sufficient Ca^{2+} ions a higher rate of inactivation of the active enzymes is observed. This effect is due to the incorporation of the reagent into the lysolecithin micelles, thus enhancing the nitration of those tyrosine residues involved in the micellar binding site of the phospholipases A_2. In order to remove polymerized protein, we subjected the reaction mixture to gel filtration on Sephadex G-75. The elution patterns obtained revealed that nitration in the absence of lysolecithin resulted in the formation of approximately 30% of dimerized protein while in the presence of lysolecithin almost no dimerization occurred. This protecting effect of lysolecithin micelles against covalent dimerization does not occur for the zymogens; the effect is specific for the active enzymes and is due to the fact that the enzymes bind to the micelles and the zymogens do not. Further purification of the nitrated monomeric protein fractions was performed by chromatography on QAE-A 25 Sephadex at pH 8, and the fractions obtained were found by analytical gel electrophoresis to be pure. Analysis of the various nitrated proteins by amino acid analyses, spectroscopic methods, and peptide mapping revealed that the invariant tyrosine-69 in all proteins as well as the variable tyrosine-123 in the porcine proteins was nitrated both in the presence and in the absence of lysolecithin micelles. It was also found that the variable tyrosine-19 was nitrated only in the active horse phospholipase A_2 when lysolecithin micelles were present. Furthermore, the presence of lipids gives rise to a considerable increase in the amount of dinitrated species in the porcine and horse phospholipases with a corresponding decrease in the amount of unmodified and mononitrated enzymes. All the nitrotyrosine-69 phospholipases exhibited considerably decreased enzymatic activity on a micellar substrate (V_{max} value of about 16% of that of the native enzymes). Upon conversion into the aminotyrosine-69 phospholipases, their enzymatic activities were restored to 55–80%. In contrast, the activity of the nitrotyrosine-19 horse phospholipase (43%) and the nitrotyrosine-123 porcine phospholipase (54%) decreased to 23 and 25%, respectively, upon conversion into their aminotyrosyl derivatives. From these results it can be concluded that the modified residues are not active site residues, but they could be involved in the binding of the enzyme to lipid–water interfaces.

Phospholipase A_2 (EC 3.1.1.4) catalyzes the specific hydrolysis of the ester bond at the C_2 position of 1,2-diacyl-3-*sn*-phosphoglycerides (van Deenen & de Haas, 1963). Ca^{2+} is required as an absolute cofactor (Pieterson et al., 1974a). The activity of the enzyme is dramatically increased when the substrate passes from a monomeric to an aggregated (micellar) form (de Haas et al., 1971; Wells, 1972).

Active site directed inhibition experiments (Volwerk et al., 1974) revealed the role of His_{48} as a catalytic site residue. Monomer phospholipid binding and Ca^{2+} binding are lost when this residue is alkylated.

Several primary structures of phospholipase A_2 have been elucidated from the following: mammalian pancreas (Evenberg et al., 1977b; Fleer et al., 1978; Puyk et al., 1977); snake venoms (Strydom, 1977); honey bee venom (Shipolini et al., 1974). Recently, the three-dimensional structure (2.4 Å) of bovine phospholipase has been reported (Dijkstra et al., 1978). The pancreatic enzymes are secreted as inactive zymogens from which the N-terminal penta- or heptapeptide is released upon limited trypsin digestion (de Haas et al., 1968). This results in the formation of the active enzyme due to the exposure of the newly formed N-terminal Ala_1. There are indications that this residue forms a salt bridge with an internal carboxylate, thus generating at the N-terminal region a certain conformation which is necessary for micellar binding (Abita et al., 1972; Pieterson et al., 1974b). Studies of the N-terminal region revealed the crucial role of Ala_1 (Slotboom & de Haas, 1975; van Dam-Mieras et al., 1975; Slotboom et al., 1977).[1]

Ultraviolet difference spectroscopic studies of the porcine enzyme (van Dam-Mieras et al., 1975) indicated that besides perturbation of the unique Trp residue located at position 3, a change in the environment of one or more Tyr residues occurs upon micellar binding. Moreover, monomer phospholipid and Ca^{2+} binding causes tyrosine and histidine perturbations (Pieterson et al., 1974b). Hence, it is of interest to examine whether or not any of these tyrosines can be selectively modified and localized.

Porcine, bovine, and equine phospholipases show considerable differences with respect to micellar, monomer phospholipid, and Ca^{2+} binding. In order to compare these phospholipases, we describe in the present paper the nitration of these isoenzymes and zymogens and the isolation of the pure nitrotyrosyl enzyme species. For this purpose the nitration procedure (Sokolovsky et al., 1966) was chosen because it has

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[1] On the basis of the 2.4-Å X-ray structure of the bovine phospholipase A_2, it has been stated (Dijkstra et al., 1978) that the terminal NH_2 group does not form a salt bridge with a carboxylate. However, one cannot preclude the possibility that the presence of 2-methyl-2,4-pentanediol in the crystallization medium (50% v/v) could give rise to some local conformational changes.

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several specific advantages. Nitrotyrosine is an excellent reporter group to detect charge and hydrophobic perturbations by using ultraviolet difference spectroscopy (Furth & Hope, 1969). Moreover, the conversion of nitrotyrosine into aminotyrosine (Sokolovsky et al., 1967) offers the possibility of successive modification of this tyrosine (Christen et al., 1971; Kenner & Neurath, 1971).

Experimental Section

Materials

Pure pig, horse, and cow (pro)phospholipases[2] were obtained as described previously (Nieuwenhuizen et al., 1974; Evenberg et al., 1977a; Dutilh et al., 1975). Chymotrypsin (treated with TLCK) was obtained from Boehringer and trypsin (treated with TPCK) was from Serva. Cyanogen bromide was purchased from Eastman, sodium dithionite was from Baker Chemicals, (2-bromoethyl)trimethylammonium bromide and dithiothreitol were from Aldrich, and *N*-acetyl-L-3-nitrotyrosine ethyl ester was from Sigma. Tetranitromethane (from Koch Light Laboratories Ltd.) was purified just before use by extracting 5 times with equal volumes of water. 1,2-Dioctanoyl-*sn*-glycero-3-phosphocholine was synthesized according to established procedures (Cubero Robles & van den Berg, 1969). Egg yolk lysolecithin was prepared by treatment of egg yolk lecithin with porcine phospholipase A_2. CM-cellulose (CM-50) (from Whatman), QAE-A 25 Sephadex, SP-C 25 Sephadex, Sephadex G-25, and Sephadex G-75 fine (from Pharmacia) were treated according to the manufacturer's instructions. Dansyl chloride and the dansylated amino acids were obtained from Sigma, and polyamide thin-layer plates were from Schleicher and Schüll (Dassel). All other chemicals were of the highest purity available and were used without further purification.

Methods

Enzymatic Assay. The enzymatic activity of phospholipase A_2 and its modified derivatives was normally determined by using the titrimetric assay procedure with egg yolk lipoprotein as a substrate (Nieuwenhuizen et al., 1974); some slight modifications were made for the horse enzyme (Evenberg et al., 1977a). In addition, the enzymatic activity of the pure modified phospholipases was determined by using micellar 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine according to the procedure described by Evenberg et al. (1977a).

Enzyme concentrations were determined from the absorbance at 280 nm, using $A_{1cm}^{1\%}$ values of 13.0 for the pig and cow enzymes (van Wezel et al., 1976; Dutilh et al., 1975) and 12.3 for the corresponding zymogens. Values of 12.3 and 11.8 were used for the horse enzyme and zymogen, respectively (Evenberg et al., 1977a).

The concentration of mononitrotyrosyl and monoaminotyrosyl proteins was determined from the absorbance at 280 nm at pH 8.0, using the $A_{1cm}^{1\%}$ values of the native proteins multiplied by a factor of 1.16 for the nitro derivative and 1.04 for the amino derivative. These factors have been derived from the increased absorbances of nitrotyrosine and aminotyrosine at 280 nm and pH 8.0 compared with free tyrosine (Sokolovsky et al., 1967).

Modification of Enzymes. The basic procedure of Sokolovsky et al. (1966) was used for the nitration of the (pro)-enzymes with some slight modifications as described under Results. Conversion of nitrotyrosyl (pro)phospholipases into their corresponding aminotyrosyl derivatives was carried out according to the method of Sokolovsky et al. (1967). To the nitrotyrosine protein solution (10 mg/mL) in a buffer containing 50 mM Tris-HCl, pH 8.0, were added very small amounts of solid sodium dithionite until the yellow color of the original nitrotyrosine completely disappeared (usually between 15 and 30 s). The protein was then immediately desalted on a Sephadex G-25 column equilibrated with 1% acetic acid, and the fractions containing the protein peak were pooled and lyophilized.

Determination of the Number of Nitrated Tyrosine Residues. The number of 3-nitrotyrosine residues in the protein preparations was determined by using the optical densities of the protein at 275 and 428 nm at pH 8.0. For calculation, eq 1 and 2 are used (Goto et al., 1971), where OD_{275} and

$$OD_{275} = \epsilon_{NP}t + (4000 - 1360)m \quad (1)$$

$$OD_{428} = 4200m \quad (2)$$

OD_{428} are the observed values for the nitrated protein and m and t represent the molar concentrations of the nitrated tyrosyl residues and the total protein, respectively. ϵ_{NP} represents the molar absorption of native (pro)phospholipases at 275 nm and could be derived from the $A_{1cm}^{1\%}$ values and the molecular weight of the protein.[3] For the molar absorptions of *N*-acetyltyrosine and *N*-acetyl-3-nitrotyrosine at 275 nm, values of 1360 and 4000, respectively, were used (Sokolovsky et al., 1966). A value of 4200 for *N*-acetyl-3-nitrotyrosine at 428 nm (Riordan et al., 1967) was used. From eq 1 and 2 the value m/t can be calculated and represents the number of nitrated tyrosyl residues per molecule of (pro)phospholipase.

$$m/t = \frac{\epsilon_{NP}OD_{428}}{4200 \times OD_{275} - 2640 \times OD_{428}}$$

Reduction of proteins was carried out at pH 8.5 in 8 M urea, 1 M Tris-HCl, pH 8.5, and a 100-fold excess of dithiothreitol. Thialamination was conducted for 20 h at 37 °C (Itano & Robinson, 1972). The reaction mixtures were desalted on Sephadex G-25 in 1% acetic acid.

Enzymatic Digestion. Reduced and thialaminated protein (5 mg) was dissolved in 0.5 mL of a 1% (w/v) solution of NH_4HCO_3. To this solution 50 μL of a freshly prepared TLCK-treated solution of chymotrypsin (1 mg/mL) was added, and incubation was carried out at 37 °C for 4 h. The digestion mixture was then lyophilized.

Isolation of NO_2-Tyr-Containing Peptides. Separation of peptides was carried out on Whatman No. 3 MM paper. High-voltage electrophoresis, using the buffer 0.62 M pyridine–0.035 M acetic acid (pH 6.5), was performed at 50 V/cm. Subsequently, descending chromatography was carried out in 1-butanol–pyridine–acetic acid–water (60:40:12:48 v/v). The paper was exposed to ammonia vapor in order to visualize the yellow NO_2-Tyr-containing peptides. The yellow peptides were cut out and eluted from the paper directly into a hydrolysis tube by a centrifugation technique (van Beynum, 1975) using 30% pyridine which was titrated to pH 9 by using concentrated ammonia. The remaining paper was stained with ninhydrin (Bennet, 1967).

[2] Abbreviations used: TNM, tetranitromethane; CNBr, cyanogen bromide; dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; TPCK, tosylphenylalanyl chloromethyl ketone; TLCK, tosyllysyl chloromethyl ketone; NT phospholipase, nitrotyrosine phospholipase; AT phospholipase, aminotyrosine phospholipase; (pro)phospholipase A_2, phospholipase A_2 and its zymogen; L-dioctanoyllecithin, 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine; ϵ_{NP}, molar absorption of native (pro)phospholipases.

[3] It is assumed that there is one nitrotyrosine per enzyme molecule. The error introduced may be of the order of 10% and thus not very significant.

Cyanogen Bromide Cleavage. One of the nitrotyrosine-containing peptides of horse phospholipase was reacted with CNBr according to Bargetzi et al. (1964) in 0.5 mL of 70% formic acid in a closed 5-mL flask at room temperature for 24 h. The molar ratio of CNBr/methionine was 100:1. The reaction mixture was lyophilized and subsequently submitted to the peptide mapping procedure as described in the preceding paragraph.

Amino Acid Analysis. Amino acid analyses were performed by the method of Spackman et al. (1958) on a Technicon T.S.M. amino acid analyzer. Suitable amounts of the nitrotyrosyl peptides were hydrolyzed in vacuum-sealed ampules with 5.6 N HCl for 24 h at 110 °C. In order to achieve a good resolution of 3-nitrotyrosine (which elutes after phenylalanine), we extended the elution time with the long-column buffer, pH 4.28, by 15 min. The relative color yield of 3-nitrotyrosine/leucine was 0.96 before and after the normal acid hydrolysis procedure. Tryptophan was determined according to the method of Liu & Chang (1971) by using a Beckman Unichrom amino acid analyzer. NH_2-terminal amino acid analysis of the nitrotyrosine-containing peptides was performed by the dansylation procedure according to Gray (1972).

Results

Effect of Nitration on the Enzymatic Activity. Porcine, bovine, and equine phospholipases A_2 were treated with tetranitromethane according to the method of Sokolovsky et al. (1966). To minimize side reactions including extensive dimer and oligomer formation (Bristow & Virden, 1978; Vincent et al., 1970), as well as modification of tryptophan (Cuatrecasas et al., 1968) and methionine (Sokolovsky et al., 1966), the following conditions were found to be satisfactory. The enzymes dissolved in a buffer (1 mg/mL) containing 0.1 M NaCl, 10 mM $CaCl_2$, and 50 mM Tris-HCl, pH 8.0, were incubated at 30 °C with a 10-fold molar excess of TNM (1% v/v in absolute alcohol). At certain time intervals, aliquots were withdrawn for determination of enzymatic activity in the egg yolk assay. As can be seen in Figure 1, initially the enzymatic activity decreases rapidly and then levels off.

In order to examine the influence on nitration of a micellar substrate, similar incubations were performed in the presence of egg yolk lysolecithin (10 mg/mL).[4] The presence of lysolecithin causes a higher initial inactivation rate as can be seen in Figure 1. It has been found that in the presence of lysolecithin micelles the solubility of TNM is increased 150-fold due to incorporation of the reagent into the apolar core of the micelles [cf. Volwerk et al. (1974)]. This explains the increased inactivation rate because the tyrosine(s) that is part of the micellar binding site encounters the relatively high concentration of TNM in the micelles.

In order to investigate which tyrosine residues are modified and to study the properties of the nitrated enzymes, we nitrated larger amounts of the three enzymes and their corresponding zymogens (100–500 mg) under the same conditions in the presence and absence of egg yolk lysolecithin (10 mg/mL). In order to prevent high local concentration of TNM, we added the reagent (1% in absolute ethanol) dropwise to the rapidly stirred solution. To avoid extensive modification, we stopped nitration after 15 min by adding acetic acid until the pH was 4.0. Subsequently, the reaction mixture was extensively dialyzed against 0.1% acetic acid at 4 °C, and the remaining lysolecithin was removed by CM-cellulose ion-exchange chromatography at pH 4.8. The bound protein was eluted with 0.5 M NaCl. After dialysis and lyophilization, in order to separate the monomer from the polymerized protein, we submitted the nitrated protein to a Sephadex G-75 column (3 × 200 cm) equilibrated with 0.1% NH_4HCO_3. Figure 2 shows

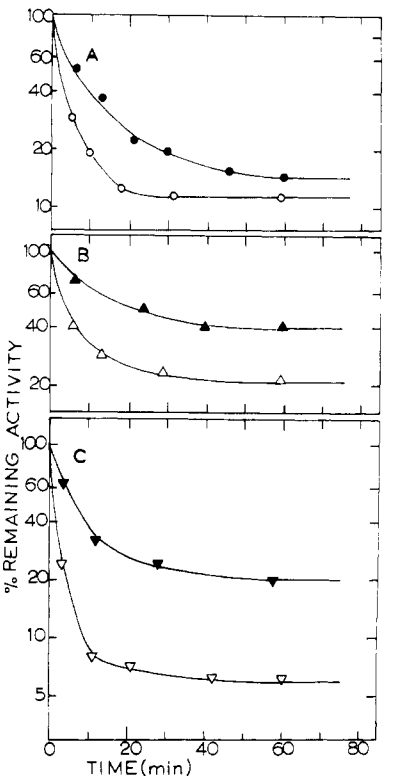

FIGURE 1: Loss of phospholipase A_2 activity as a function of time upon addition of tetranitromethane. The following are the experimental conditions. To a solution (2 mL) of equine (A), porcine (B), and bovine (C) phospholipase A_2 (1 mg/mL) in a buffer containing 0.1 M NaCl, 10 mM $CaCl_2$, and 50 mM Tris-HCl, pH 8.0, was added 20 μL of a solution of 1% tetranitromethane in ethanol. Incubation was performed at 30 °C. At suitable time intervals, aliquots (25–200 μL) were withdrawn and the enzymatic activity was assayed by using the egg yolk assay. (●) Equine, (▲) porcine, and (▼) bovine phospholipases. Similar incuations were made in the presence of egg yolk lysolecithin (10 mg/mL): (○) horse, (△) pig, and (▽) ox phospholipases. For the bovine enzyme in the presence of lysolecithin 50 mM $CaCl_2$ was used.

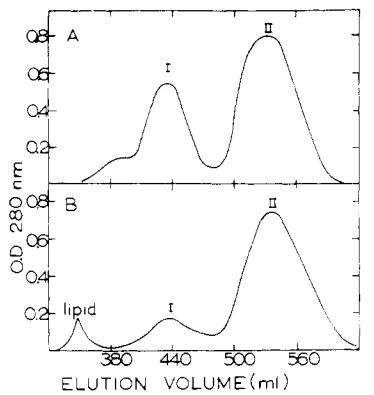

FIGURE 2: Gel filtration patterns of nitrated horse phospholipase A_2 in 1% NH_4HCO_3, pH 8.0, from a column (3.5 × 200 cm) of Sephadex G-75. The following are the experimental conditons. Nitrated phospholipase (200 mg) in the absence (A) and presence (B) of egg yolk lysolecithin (as outlined under Results) was loaded onto the column in 3 mL of the elution buffer. The flow rate was set at 100 mL/h. Peaks I represent dimeric enzymes; peaks II are monomeric enzymes.

[4] In contrast to the horse and porcine phospholipases A_2, the bovine enzyme requires higher $CaCl_2$ concentrations than 10 mM for optimal binding to lysolecithin micelles at pH 8. Therefore, the nitration of the bovine enzyme was performed in the presence of 50 mM $CaCl_2$.

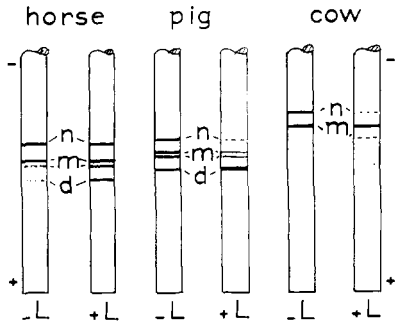

FIGURE 3: Analytical polyacrylamide gel electrophoresis patterns of the monomeric nitro equine, porcine, and bovine phospholipase fractions, nitrated in the absence or presence of egg yolk lysolecithin. The following are the experimental conditions. The proteins (20 μg) were loaded onto 7.5% polyacrylamide gels (pH 8.5). Electrophoresis was carried out at 200 V and 5 mA/gel for 35 (equine enzyme), 60 (porcine enzyme), and 90 min (bovine enzyme). n represents not-modified (native) enzyme and m represents mononitrated enzyme species, whereas d represents dinitrated enzyme species.

a typical elution pattern of horse phospholipase A_2 nitrated in the absence (Figure 2A) or presence (Figure 2B) of lipid. Making use of the formula

$$\log M = 5.624 - 0.752\frac{V_e}{V_0}$$

which is valid for Sephadex G-75 (Whitaker, 1963), the apparent molecular weight for peak I was found to be 28 000 ± 2000 and for peak II was 14 000 ± 1000. This clearly demonstrates peak I to be a dimer and peak II to be the monomeric enzyme. It is obvious that lysolecithin micelles give considerable protection against covalent dimerization of phospholipase A_2 (Figure 2B). Similar elution patterns were obtained for the nitrated pig and ox enzymes. The dimer enzyme fractions only show about 1% of the enzymatic activity of that of the native enzymes and have not been studied further. Nitration of the zymogens in the absence or presence of lysolecithin resulted in essentially the same elution patterns as shown in Figure 2A. The protecting effect of lysolecithin against covalent dimerization is specific for the active enzyme and is due to the fact that the enzyme binds to the micelles and the zymogen does not.

The monomer fractions were analyzed by polyacrylamide gel electrophoresis at pH 8.5. As can be seen from Figure 3, nitration of the various enzymes gives rise to several mono- and dinitrated species, and the relative amounts are dependent on the presence or absence of lysolecithin micelles. Pilot experiments using ion-exchange chromatography on QAE-A 25 Sephadex at different pH values, column lengths, and salt gradients finally revealed the optimal conditions for separation which are summarized in the legend of Figure 4. The first peaks n represent unmodified enzymes as judged by the absence of absorption at 428 nm and by the original specific activity. With increasing salt concentration, material represented by peaks m and d are eluted, showing an incorporation of one and two nitrotyrosine residues per mol of enzyme, respectively. It is obvious from Figure 4 (A and B) that nitration of horse phospholipase in the presence of lipid results in an additional mononitrated enzyme species (Figure 4B, peak m_{II}). In contrast, the porcine phospholipase A_2 nitrated in the absence of lipids contains two mononitrated enzyme species (Figure 4C, peaks m_I and m_{II}) while the presence of lipids results almost exclusively in formation of the dinitrated analogue (Figure 4D, peak d). In the case of bovine phospholipase A_2, predominantly one tyrosine per mol of phospholipase is nitrated both in the absence and in the presence

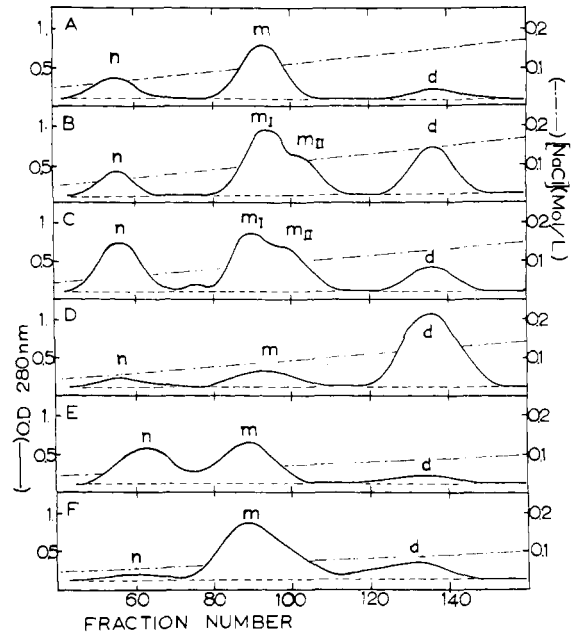

FIGURE 4: Elution patterns of monomeric nitrated horse, pig, and ox phospholipase fractions from QAE-A 25 Sephadex at pH 8.0. The following are the experimental conditions. QAE-A 25 Sephadex columns (2.3 × 140 cm), equilibrated at 4 °C with 5 mM Tris-HCl, pH 8.0, were loaded with 300 mg of the monomeric nitrated enzyme fractions in the same buffer and developed with 10 L of a linear salt gradient reaching 0.25 M NaCl for the equine enzyme (A,B), 0.2 M NaCl for the porcine enzyme (C,D), and 0.15 M NaCl for the bovine enzyme (E,F). Fractions of 30 mL were collected at a flow rate of 100 mL/h. Peaks n represent native enzymes and peaks m are mononitrated species, whereas peaks d represent the dinitrated enzymes. A, C, and E refer to fractions obtained in the absence of lysolecithin micelles while B, D, and E refer to fractions obtained in the presence of lysolecithin micelles.

of lipid. However, the yield of mononitrotyrosine bovine enzyme is much greater in the presence of lipid (Figure 4E,F). The mononitrated species of horse and pig phospholipase were rechromatographed by using the same conditions as above and were tested for homogeneity by analytical polyacrylamide gel electrophoresis at pH 8.5.[5] It was found that all nitrotyrosine proteins were pure except for the mononitrated porcine phospholipase represented by fraction m_{II} (Figure 4C). It was not possible to obtain the latter protein in a form completely free from a slight contamination with the mononitrated phospholipase represented by fraction m_I (Figure 4C). The elution patterns of the nitrated zymogens were essentially the same as those shown for the active enzymes nitrated in the absence of lipid (Figure 4A,C,E).

Localization of the Nitrated Tyrosines. Pure nitrated protein (5 mg of each) was reduced, thialaminated, and subsequently digested with chymotrypsin. Peptide mapping of the digest was performed as described under Methods. Sufficient amounts of nitrotyrosine-containing peptides (10–20 nmol) were selected for amino acid analysis and about 10 nmol was selected for determination of the N-terminal amino acid. Table I summarizes the data obtained. For the sake of clarity Figure 5 shows the primary structures of equine, porcine, and bovine (pro)phospholipase A_2, thus facilitating the recognition of the several nitrotyrosine-containing peptides. These results clearly demonstrate that tyrosine-69, invariant in all enzymes, is nitrated both in the absence and in the presence of lipid. In addition, the variable tyrosine-19 in horse phospholipase is nitrated only in presence of lysolecithin micelles. The

[5] In the mononitrated enzymes no oxidized methionines could be detected and tryptophan was not affected.

Table I: Amino Acid Composition[a] and N-Terminal Amino Acid Residues of the Different Nitrotyrosine-Containing Peptides

amino acid	origin of peptide:[b] residue no:	equine (A-m, B-m_I) 64–69	equine (B-m_{II})[d] 9–19	porcine (C-m_I) 64–69	porcine (C-m_{II}) 119–123	bovine (E-m, F-m) 65–69
		mol/mol of peptide[a]				
aspartic acid + asparagine		1.9 (2)	1.0 (1)	2.2 (2)	1.1 (1)	2.0 (2)
threonine			1.0 (1)		1.0 (1)	
serine			1.0 (1)			
glutamic acid + glutamine			0.6 (1)			
proline		1.1 (1)	1.9 (2)	1.2 (1)		1.0 (1)
valine		1.1 (1)		1.0 (1)		0.9 (1)
isoleucine			1.2 (1)			
leucine		1.0 (1)		1.0 (1)		
tyrosine		–	–	–	–	–
NO_2-tyrosine		1.0 (1)	1.1 (1)	0.8 (1)	0.9 (1)	1.1 (1)
lysine[c]			+ (1)		2.4 (2)	
cysteine[c]			+ (1)			
N-terminal amino acid		leucine	isoleucine	leucine	aspartic acid (or asparagine)	valine

[a] Expected values are in parentheses. [b] The nomenclature of the peptides refers to the corresponding mononitrated phospholipases as indicated in Figure 4. [c] Thialaminated cysteine is incompletely separated from lysine on the short column of the amino acid analyzer. [d] Obtained after CNBr cleavage of the original chymotryptic peptide.

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   -7                       1                           10
a) <Glu-Glu-Gly-Ile-Ser-Ser-Arg-Ala-Leu-Trp-Gln-Phe-Arg-Ser-Met-Ile-Lys-Cys-Ala-Ile-Pro-Gly-
b)   .  Ala  .  Leu Asn  .   .   .   .   .   .   .  Asn Gly  .   .   .   .  Lys  .   .  Ser
c)  Glu Asn  .   .   .  Pro  .   .  Val  .   .   .   .   .   .   .  Gln  .  Thr  .   .  Asn

                20                              30
   Ser-His-Pro-Leu-Met-Asp-Phe-Asn-Asn-Tyr-Gly-Cys-Tyr-Cys-Gly-Leu-Gly-Gly-Ser-Gly-Thr-Pro-
    .  Glu  .   .  Leu  .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .
    .  Lys  .  Tyr Leu Glu  .   .  Asp  .   .   .   .   .   .   .   .   .   .   .   .   .

        40                              50
   Val-Asp-Glu-Leu-Asp-Arg-Cys-Cys-Glu-Thr-His-Asp-Asn-Cys-Tyr-Arg-Asp-Ala-Lys-Asn-Leu-Asp-
    .   .  Asp  .   .   .   .   .  Gln  .   .   .   .   .   .   .  Lys Gln  .   .  Lys  .   .
    .   .   .   .   .  Ala  .   .  Gln Val  .   .   .   .   .   .  Thr Gln  .   .  Glu  .  Ser

   60                                  70                                      80
   Ser-Cys-Lys-Phe-Leu-Val-Asp-Asn-Pro-Tyr-Thr-Glu-Ser-Tyr-Ser-Tyr-Ser-Cys-Ser-Asn-Thr-Glu-
    .   .   .  Val  .   .   .   .   .   .   .  Asn Asn  .   .   .   .   .   .   .  Asn  .
    .   .  Arg  .   .   .   .   .   .   .   .   .   .   .   .  Lys Phe  .   .   .  Gly  .   .

                                    90                                  100
   Ile-Thr-Cys-Asn-Ser-Lys-Asn-Asn-Ala-Cys-Glu-Ala-Phe-Ile-Cys-Asn-Cys-Asp-Arg-Asn-Ala-Ala-
    .   .   .  Ser  .  Glu  .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .
   Val  .   .  Ser Asp  .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .

                            110                                 120
   Ile-Cys-Phe-Ser-Lys-Ala-Pro-Tyr-Asn-Lys-Glu-His-Lys-Asn-Leu-Asp-Thr-Lys- 0 -Lys-Tyr-Cys-
    .   .   .   .   .   .  Val  .   .   .   .   .   .   .   .   .  -0-  .  -0-  .  Asn  .
    .   .   .   .   .   .   .   .   .  Pro  .  Asn  .   .   .   .  Ser  .  Arg  .  Ala  .
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FIGURE 5: Amino acid sequence of prophospholipase A_2 from pig (upper), horse (lower), and ox (middle). Deletions (-0-) have been assigned to achieve maximal homology; (·) represents an amino acid residue identical with that given for the porcine enzyme.

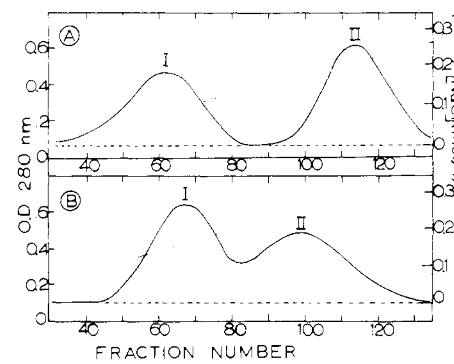

FIGURE 6: Elution patterns of aminotyrosyl phospholipase A_2 from horse (A) and from pig (B) on SP Sephadex C-25 at pH 5.0. The following are the experimental conditions. SP Sephadex C-25 columns (1.5 × 100 cm), equilibrated at 4 °C with 5 mM sodium acetate, pH 5.0, were loaded with 150 mg of monoaminotyrosyl horse (A) and pig (B) phospholipase in the same buffer and developed with 2 L of a linear salt gradient reaching 0.4 M NaCl in the same buffer. Fractions of 15 mL were collected at a flow rate of 50 mL/h.

variable tyrosine-123 of the porcine enzyme is nitrated in the absence and presence of lipid.

Purification of Aminotyrosine (Pro)phospholipases on a Preparative Scale. In contrast to the laborious isolation of the pure mononitrated horse and porcine phospholipases A_2, the resolution of their aminotyrosine analogues by ion-exchange chromatography on SP-C 25 Sephadex at pH 5 proved to be much easier. As can be seen from Figure 6A, aminotyrosine-19 horse phospholipase (peak I) is completely separated from the aminotyrosine-69 enzyme (peak II). The separation of aminotyrosine-123 pig phospholipase (Figure 6B, peak I) and the aminotyrosine-69 enzyme (peak II) is also very satisfactory. These aminotyrosyl enzymes were pure as judged by polyacrylamide gel electrophoresis at pH 4.3 and 8.5.

Enzymatic Activities of the Nitrotyrosyl and Aminotyrosyl Enzymes. The enzymatic activity of the purified nitrotyrosyl and aminotyrosyl enzymes was determined by using the egg yolk assay (pH 8) and the synthetic substrate 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine. The results listed in Table II show that all nitrated enzymes exhibit a substantial decrease of enzymatic activity. This is most evident for the nitrotyrosine-69 enzymes. The conversion of the latter into their aminotyrosyl derivatives results in a striking increase in enzymatic activity. However, conversion of the horse nitrotyrosine-19 and porcine nitrotyrosine-123 phospholipases into their aminotyrosine species results in a considerable decrease in their enzymatic activities. These results indicate that the charges on the modified tyrosines are most important for enzymatic activity.

Discussion

The three-dimensional structure of bovine pancreatic phospholipase A_2 which has recently been elucidated (Dijkstra et al., 1978) enables us to discriminate between tyrosines exposed and tyrosines not exposed to solvent. As can be seen in the stereo diagram (Figure 7), the invariant tyrosine-69 is fully exposed. The above information together with the knowledge of the primary structures of horse and pig phospholipase can be used to predict that the variable tyrosine-19 of horse phospholipase and the variable tyrosine-123 of pig phospholipase are exposed to solvent whereas the other tyrosines (25, 28, 52, 73, 75 and 111) have little or no possible access to solvent (cf. also Figure 7). The distribution of exposed tyrosines on the protein surface and the degree of aggregation (which is dependent on protein concentration and ionic strength) influence the polymerization side reaction during nitration (Williams & Lowe, 1971; Bruice et al., 1968;

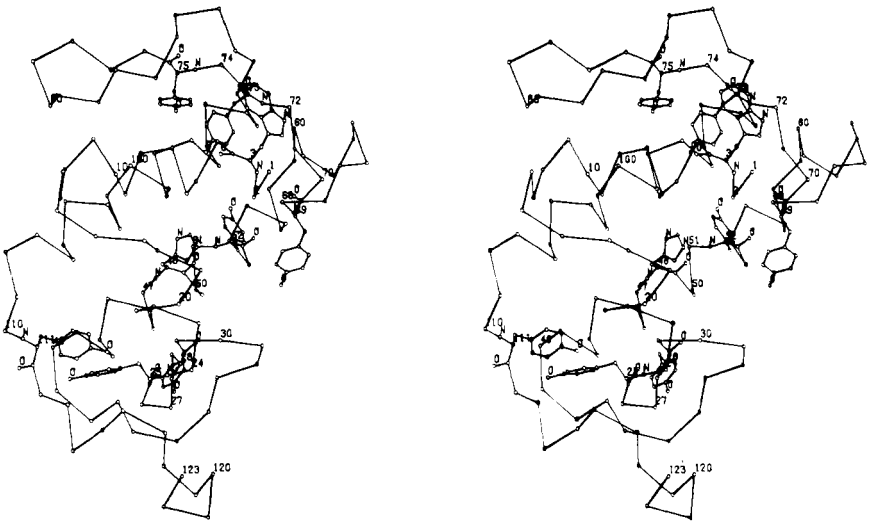

FIGURE 7: Stereo diagram showing C_α carbon atoms and the position of the side chains of the tyrosine and tryptophan residues and histidine-48 in bovine phospholipase A_2 at 2.0-Å resolution.

Table II: Enzymatic Activities of Nitrotyrosine (NT) and Aminotyrosine (AT) Porcine, Equine, and Bovine Phospholipases

	sp act. (egg yolk assay)		V_{max}^{app} [a] (dioctanoyl lecithin assay)	
	μequiv min^{-1} mg^{-1}	%	μequiv min^{-1} mg^{-1}	%
equine phospholipase A_2	2000	100	16500	100
equine NT_{19}PLA	860	43	11200	67
equine AT_{19}PLA	455	23	3300	20
equine NT_{69}PLA	350	17.5	1950	11
equine AT_{69}PLA	1000	55	8580	52
equine di$NT_{19}NT_{69}$PLA	136	7	1170	7
porcine phospholipase A_2	1400	100	5600	100
porcine NT_{69}PLA	220	15.7	600	11
porcine AT_{69}PLA	950	68	3040	54
porcine NT_{123}PLA	760[b]	54[b]	2350[b]	42[b]
porcine AT_{123}PLA	350	25	1680	30
porcine di$NT_{69}NT_{123}$PLA	140	10	440	8
bovine phospholipase A_2	70	100	5530	100
bovine NT_{69}PLA	11.6	16	840	15
bovine AT_{69}PLA	57.5	80	3360	61

[a] These V_{max}^{app} values have been determined at pH 8 and 48 °C (0.1 M NaCl and 50 mM $CaCl_2$) by using 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine by a double-reciprocal plot of the activities and substrate concentrations (Lineweaver & Burk, 1934). Based on the kinetic model of lipolysis by Verger et al. (1973), these V_{max}^{app} values are lower limit values because it cannot a priori be taken for granted that the monomer binding site is fully saturated under the conditions used (see also the following paper). [b] These values are less certain due to contamination by about 20% NT_{69} phospholipase.

Vincent et al., 1970). This undesired side reaction could be minimized by using low protein concentration (1 mg/mL) and moderate ionic strength. Nevertheless, some remarkable differences have been observed upon nitrating the various phospholipases. In the absence of egg yolk lysolecithin, nitration resulted in the formation of approximately 30% dimerized protein, while almost no dinitrated monomeric protein was formed. On the other hand, upon nitration in the presence of lysolecithin micelles, almost no dimerized protein was formed, but sizable amounts of dinitrated monomeric proteins were produced (Figures 2 and 3). The increased concentration of tetranitromethane in the lysolecithin micelles caused an increase in the rate of nitration of the exposed tyrosine residues (Figure 1). Both nitrated and native enzyme molecules may remain bound to the micelles, which most probably results in a decreased diffusion rate, and encounter chance limiting the unmodified tyrosine to enter cross-linking reactions. The results shown in Figures 1 and 2 strongly support this hypothesis. Moreover, nitration of the zymogens, which do not bind micellar substrate, did not show the protective effect of lysolecithin micelles against covalent dimerization of the protein, providing additional evidence for the specificity of this protection. From the finding that all nitrated phospholipases and aminotyrosine phospholipases described have inherent residual enzymatic activity (Table II), it can be concluded that the modified tyrosine residues are not involved in the active site. One must keep in mind, however, that the enzymatic activity of lipolytic enzymes is a function of micellar binding, Ca^{2+} binding, and monomer phospholipid binding. Therefore, the influence of the modified tyrosines upon these functions has been extensively studied and will be reported in the accompanying paper.

Remarkable is the nitration of Tyr_{19} in horse phospholipase A_2 in the presence of lysolecithin (Figure 4B, peak m_{II}), which does not occur for the zymogen. This can be explained in two ways. First, as already stated, the increased solubility of tetranitromethane in the micelles will result in the nitration of those tyrosines that are part of the micellar binding site of phospholipase. The considerably increased nitration of tyrosine-69 in bovine phospholipase in the presence of lysolecithin (Figure 4F, peak m) also strongly supports this idea. Second, micellar binding is known to induce a conformational change in phospholipase A_2. This change may result in a different position of tyrosine-19 being more favorable for attack by TNM.

Finally, it has to be mentioned that reaction of bovine and porcine (pro)phospholipases with an equimolar quantity of iodine also results in the specific monoiodination of Tyr_{69}. In addition, Tyr_{123} in the porcine proteins was also partially monoiodinated (Slotboom et al., 1978). Apparently the exposed Tyr_{69} and Tyr_{123} residues are in a particular environment, making them readily susceptible toward both nitration and iodination. In this respect, it is remarkable that very close to Tyr_{69} as well as to Tyr_{123} (which is Asn_{123} in the bovine enzyme) a lysine residue is present as found upon inspection of the X-ray model.

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