Comparative Studies of Tyrosine Modification in Pancreatic Phospholipases. 2. Properties of the Nitrotyrosyl, Aminotyrosyl, and Dansylaminotyrosyl Derivatives of Pig, Horse, and Ox Phospholipases A_2 and Their Zymogens†

H. Meyer,* W. C. Puijk, R. Dijkman, M. M. E. L. Foda-van der Hoorn, F. Pattus,‡ A. J. Slotboom, and G. H. de Haas

ABSTRACT: Specifically nitrated phospholipases A_2 and the corresponding zymogens from pig, horse, and ox pancreas have been used to investigate the role of particular tyrosine residues in phospholipid and Ca^{2+} binding. Ultraviolet difference spectroscopy allowed direct binding studies with *n*-alkylphosphocholines as the substrate analogues. After reduction and dansylation of the nitrated enzymes, the interaction with phospholipids could also be monitored by fluorescence spectroscopy. Due to the considerable enzymatic activity of the nitrotyrosine, aminotyrosine, and dansylaminotyrosine phospholipases, their action on monomolecular surface layers of 1,2-didecanoyl-*sn*-glycero-3-phosphocholine as substrate was investigated. From this study it is concluded that the invariant tyrosine-69 in horse, pig, and ox phospholipases A_2 and the variable tyrosine-19 in the horse enzyme are part of the previously proposed interface recognition site [van Dam-Mieras, M. C. E., Slotboom, A. J., Pieterson, W. A., & de Haas, G. H. (1975) *Biochemistry 14*, 5387–5394] and that tyrosine-123 in the porcine enzyme is not involved in binding to lipid–water interfaces. In contrast to the modified phospholipases A_2, the corresponding nitrotyrosine, aminotyrosine, and dansylaminotyrosine zymogens do not bind to micellar substrate analogues. Monomer phospholipid binding (as monitored by ultraviolet difference spectroscopy) induces a strong hydrophobic perturbation of nitrotyrosine-69 and -19, and again the microenvironment of nitrotyrosine-123 is not changed. Because of the lower p*K* of nitrotyrosine as compared to tyrosine, measurements were also performed at pH 8.0 where nitrotyrosine is negatively charged. Both monomer binding and penetrating capacity of nitrotyrosine-69 phospholipase are severely distorted, probably because of charge repulsion between the negatively charged nitrotyrosine and the phosphate moiety of the phospholipid. Finally, it could be shown by ultraviolet difference spectroscopy that binding of the essential cofactor Ca^{2+} to the tyrosine-69 nitrated phospholipases results in a conformational change influencing the microenvironment of tyrosine-69. Horse nitrotyrosine-19 phospholipase and pig nitrotyrosine-123 phospholipase are not perturbed by Ca^{2+} binding. These results are in agreement with the recently proposed three-dimensional structure of the bovine phospholipase A_2 [Dijkstra, B. W., Drenth, J., Kalk, K. H., & Vandermaelen, P. J. (1978) *J. Mol. Biol. 124*, 53–60].

Nitration of equine, porcine, and bovine pancreatic (pro)phospholipases A_2[1] yields several differently modified enzyme species as described in the preceding paper. It was shown that the invariant tyrosine-69 in the above proteins is nitrated both in the absence and in the presence of egg yolk lysolecithin. Under these conditions the variable tyrosine-123 in porcine (pro)phospholipase is also nitrated. On the other hand, the variable tyrosine-19 in horse phospholipase is nitrated only in the presence of lysolecithin.

The unique properties of nitrotyrosine facilitate different approaches in studying the environmental changes of these residues upon substrate and metal ion binding. The p*K* of the phenolic-OH group of nitrotyrosine, which is 7.2, is very sensitive toward charge effects (Sokolovsky et al., 1967). Also, hydrophobic changes can be monitored by using the visible absorption band at 428 nm (Furth & Hope, 1969; Cuatrecasas et al., 1968). Conversion, using sodium dithionite, of nitrotyrosine into aminotyrosine generates an aromatic amino group with a p*K* below 5 whereas the p*K* of the phenolic OH is almost restored to the original value (Sokolovsky et al., 1967). This property enables us to introduce specifically a covalently attached dansyl group onto the aminotyrosines. This dansyl group serves as a very sensitive indicator for detecting changes in the polarity of a protein (Kenner & Neurath, 1971).

The aim of the present paper is to describe the interaction of nitrotyrosine, aminotyrosine, and dansylaminotyrosine derivatives of the various (pro)phospholipases A_2 with micellar and monomer phospholipid and Ca^{2+} ions. Several techniques, including ultraviolet difference and fluorescence spectroscopy and phospholipid monolayer kinetics, have been used in order to delineate the role of the modified tyrosines in the enzymatic process.

Experimental Section

Materials

Most of the materials have been described in the preceding paper (Meyer et al., 1979). The model compounds *N*-

† From the Laboratory of Biochemistry, State University of Utrecht, Transitorium 3, "De Uithof", Padualaan 8, Utrecht, The Netherlands. *Received December 19, 1978.* 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).

‡ Present address: Institut für Biochemie, Universität Bern, 3012 Bern, Switzerland.

[1] Abbreviations used: dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; dansyl-OH, 5-(dimethylamino)naphthalene-1-sulfonic acid; NT(pro)PLA, nitrotyrosine (pro)phospholipase A_2; AT(pro)PLA, aminotyrosine (pro)phospholipase A_2; Dns-AT(pro)PLA, dansylaminotyrosine (pro)-phospholipase A_2; cmc, critical micellar concentration; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); (pro)phospholipase A_2, phospholipase A_2 and its zymogen; L-α-didecanoyllecithin, 1,2-didecanoyl-*sn*-glycero-3-phosphocholine.

0006-2960/79/0418-3589$01.00/0

acetyl-3-aminotyrosine ethyl ester and *N*-acetyl-3-dansylaminotyrosine ethyl ester were prepared from *N*-acetyl-3-nitrotyrosine ethyl ester (from Sigma) as described by Kenner (1971). *n*-Alkylphosphocholines were prepared as described by van Dam-Mieras et al. (1975). 1,2-Didecanoyl-*sn*-glycero-3-phosphocholine was prepared as described by Cubero Robles & van den Berg (1969). 1-Bromo-2-octanone was prepared according to the procedure as described by Visser et al. (1971) and Mangold (1973). All other chemicals were of the highest purity available.

Methods

Ultraviolet Absorption (Difference) Spectroscopy. Spectra were recorded on an Aminco DW-2A spectrophotometer, equipped with a 31-step base-line correction accessory. When the interaction of enzyme–phospholipid was studied, tandem cells (2 × 1 cm path length) were used because of the absorption of the phospholipid in the ultraviolet region (van Dam-Mieras et al., 1975). Lipid was added to the protein solution of the sample compartment and to the buffer solution of the reference compartment. The same amount of buffer was added to the protein solution of the reference compartment to correct for the dilution of the chromophores. Protein concentrations never exceeded 70 μM in order to prevent deviations from Beer's law. Ca^{2+} titrations were performed in cells of 1-cm path length according to the method described by Pieterson et al. (1974a). Titrations were performed at 25 °C in buffer containing 0.1 M NaCl and 50 mM sodium acetate, pH 6.0, or 0.1 M NaCl and 50 mM Tris-HCl, pH 8.0.

Spectrophotometric pH Titration of Nitrotyrosine (Pro)phospholipases. Protein samples (1 mg/mL) in 0.1 M NaCl were titrated in 1-cm cuvettes with minute volumes of 10% ammonia or NaOH. pH measurements were made directly in the cuvette using a Radiometer pH-28 pH meter equipped with a combined electrode, Model GK 2321 C. Titration between pH 4 and pH 11 was followed by monitoring the absorbance at 428 nm.

Fluorescence Measurements of Dansylaminotyrosine (Pro)phospholipases. Fluorescence spectra were measured at 25 °C with a Perkin-Elmer MPF 3 spectrofluorometer using 1-cm cells and a thermostated cell holder. Excitation and emission slit widths were set at 5 nm. In order to prevent internal quenching effects, we never allowed the protein concentration to exceed 14 μM. Titration with *n*-hexadecylphosphocholine was done in 3-mL cuvettes containing 2.0 mL of the protein solution in 0.1 M NaCl and 50 mM sodium acetate, pH 6.0.

Modification of the Enzymes with 1-Bromo-2-octanone. The basic procedure described previously for *p*-bromophenacyl bromide (Volwerk et al., 1974) was used with 1-bromo-2-octanone for alkylation of the active site histidine-48 in the enzymes. A modification was to carry out the procedure in 0.1 M cacodylate buffer, pH 6.0, at 40 °C with a 10-fold molar excess of the reagent. The reagent was added as a 10% solution in ethanol. The specific activity was monitored during the inactivation until a value of less than 1% in the egg yolk assay was reached. The reaction mixture was desalted on a Sephadex G-25 column in 1% acetic acid.

Preparation of Dansylaminotyrosine (Pro)phospholipases. Aminotyrosine (pro)phospholipase A_2 (10 mg) in 1 mL of a buffer containing 0.2 M sodium acetate, pH 5.0, was reacted for 1 h at room temperature with 50 μL of a solution of 0.1 M dansyl chloride in acetonitrile. Subsequently, the fluorescent protein solution was desalted in the dark on a Sephadex G-25 column in 1% acetic acid. The elution of the protein was monitored with an ultraviolet lamp at 350 nm. The desalted protein was lyophilized in the dark and kept at −20 °C until use. As a control for the specificity of the procedure, a sample of native phospholipase A_2 was reacted under the same conditions, resulting in a protein completely devoid of dansyl fluorescence and retaining its original specific activity.

Protein Concentrations. The concentrations of native phospholipase A_2 and its nitrotyrosine and aminotyrosine derivatives were spectrophotometrically determined as described in the preceding paper. The concentrations of the dansylaminotyrosyl phospholipases were determined by measuring their absorbances at 335 nm using the molar absorption of dansylaminotyrosine (4600 M^{-1} cm^{-1}) as described by Kenner & Neurath (1971).

Monolayer Experiments. The zero-order trough with two compartments and the surface barostat technique used to measure substrate hydrolysis were identical with those described by Verger & de Haas (1973). Calculation of the induction time (τ) was performed as described previously by Verger et al. (1973). Surface pressure (π) was measured by the Wilhelmy plate method, using a thin platinum plate (perimeter 3.94 cm) attached to a Beckman R 1I C electromicrobalance, Model LM 600.

Results

Micellar Binding of the Modified Phospholipases as Determined by (Difference) Absorption Spectroscopy. As described previously, ultraviolet difference spectra obtained by titrating porcine phospholipase A_2 with the nondegradable substrate analogue *n*-hexadecylphosphocholine indicated a change in the microenvironment of the single tryptophan residue and of one or more tyrosine residue(s) (van Dam-Mieras et al., 1975). The zymogen does not show this effect, proving that the difference absorbance signals originate from micellar binding to the active enzyme.

The availability of various pure well-defined mononitrotyrosyl enzymes enables us to monitor changes in their microenvironments due to the unique properties of nitrotyrosine. Together with the presentation of these results, some adequate spectroscopic parameters of the model compound *N*-acetyl-3-nitrotyrosine ethyl ester will be given. The visible absorption spectrum of this compound exhibits a maximum at 428 nm which shifts to 358 nm upon acidification (Sokolovsky et al., 1966). The difference spectrum of this model compound (pH 8/pH 6) shows a main maximum at 431 nm (Figure 1A). The difference spectrum of horse nitrotyrosine-69 phospholipase A_2 (pH 8/pH 6) (Figure 1B) closely resembles that of the model compound, thus proving that the pH change induces a pure charge effect on this nitrotyrosine residue. The absorbance at 428 nm as a function of pH enables calculation of the p*K* of the phenolic-OH group of nitrotyrosine, which is 7.2 for the model compound (Sokolovsky et al., 1967). However, the p*K* of the various nitrotyrosine residues in (pro)phospholipase is influenced by neighboring amino acid residues (Table I). Moreover, the p*K* can be strongly influenced by substrate binding (Table I).

In addition to the pure charge effects, a hydrophobic perturbation can be monitored. Figure 1C shows the difference spectrum of the model compound in 50% dioxane at pH 6.0. This spectrum is characterized by a minimum at 419 nm. The blue shift of this minimum (12 nm) indicates the hydrophobic perturbation of nitrotyrosine.

Generally, solvent perturbation of a chromophore results in an absorption spectrum which is red-shifted as compared to that of the chromophore in water. This results in a difference spectrum in which the minimum is blue-shifted and

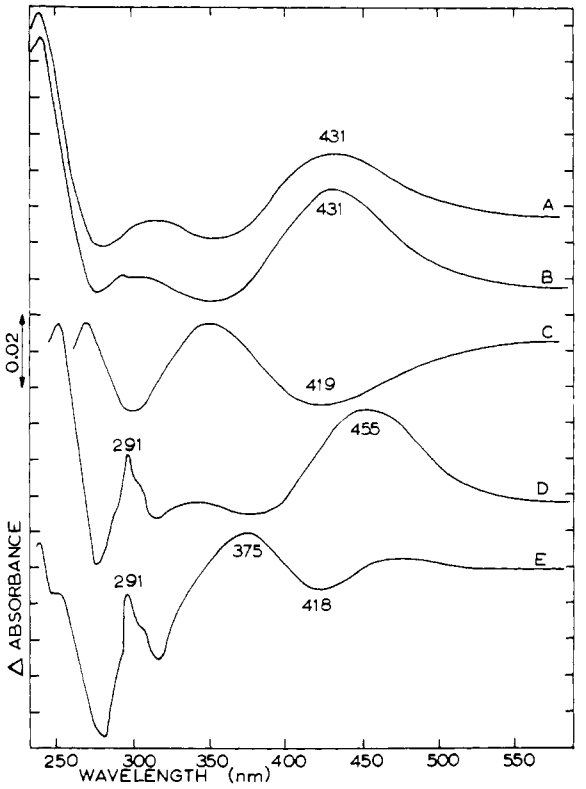


FIGURE 1: Ultraviolet difference spectra produced by *N*-acetyl-3-nitrotyrosine ethyl ester and several nitrotyrosine phospholipases upon charge and solvent perturbation. The following are the experimental conditions. Curve A: *N*-acetyl-3-nitrotyrosine ethyl ester (25 μM) in 0.1 M NaCl and 50 mM sodium acetate, pH 6.0. After equilibration and base-line correction, the sample compartment only was brought to pH 8.0 with solid Tris, and the difference spectrum was recorded. Curve B: horse nitrotyrosine-69 phospholipase A_2 (40 μM) treated in the same way as described for curve A. Curve C: *N*-acetyl-3-nitrotyrosine ethyl ester (50 μM) in 0.2 M Pipes, pH 6.0; 50% dioxane (v/v) was present in the sample compartment and in the buffer solution of the reference compartment. Curve D: pig nitrotyrosine-69 phospholipase A_2 (40 μM) in 0.1 M NaCl and 50 mM sodium acetate, pH 6.0, in presence of 5 mM *n*-hexadecylphosphocholine as described under Methods. Curve E: horse nitrotyrosine-19 phospholipase A_2 (40 μM) treated in the same way as described for curve D.

the maximum is red-shifted as compared to the absorption maximum (Donovan, 1970). Often, however, a change in absorption accompanies a hydrophobic perturbation so that either the negative or the positive part of the difference spectrum may be diminished (Donovan, 1970).

Keeping these possibilities in mind, we have titrated the nitrotyrosine phospholipases with *n*-hexadecylphosphocholine at pH 6.0 as described under Methods. Figure 1D shows the difference spectrum of porcine NT_{69}PLA in the presence of 5 mM phospholipid. Besides the expected tryptophan perturbation at 291 nm (van Dam-Mieras et al., 1975), the spectrum is dominated by a nitrotyrosine perturbation. The maximum at 455 nm, indicating a large red shift of 24 nm, suggests a rather apolar environment of this nitrotyrosine. A concomitant decrease of the p*K* is observed upon spectrophotometric pH titration (from 7.1 to 6.5; Table I). For the sake of clarity, the spectra belonging to the lower lipid concentrations are omitted in Figure 1D, but they have been used for calculation of the dissociation constants by plotting the amplitudes vs. the phospholipid concentrations in a double-reciprocal way according to the method of Lineweaver & Burk (1934). For comparison the native phospholipases and the aminotyrosyl derivatives have also been titrated, and the results obtained are summarized in Table I. The K_D value of porcine NT_{69}PLA (Table I) suggests improved micellar

Table I: Some Properties of Horse, Pig, and Ox Nitrotyrosine, Aminotyrosine, and Dansylaminotyrosine Phospholipases A_2 upon Interaction with *n*-Hexadecylphosphocholine at pH 6.0 and 25 °C as Determined by Ultraviolet Difference Spectroscopy and Spectrometric pH Titrations

enzyme derivative	λ_{max} of the difference absorption (nm)	dissociation constant K_D (mM) (± 10%)	p*K* of nitrotyrosine (± 0.05) without lipid	p*K* of nitrotyrosine (± 0.05) 50 mM lipid
horse phospholipase A_2 (PLA)		5.0		
horse NT_{19}PLA	418	0.5	7.40	8.10
horse AT_{19}PLA		1.5		
horse Dns-AT_{19}PLA	355	0.27		
horse NT_{69}PLA	455	2.7	7.20	6.90
horse AT_{69}PLA		6.6		
horse Dns-AT_{69}PLA	355	0.5		
pig phospholipase A_2 (PLA)		1.8		
pig NT_{69}PLA	452	0.9	7.10	6.50
pig AT_{69}PLA		1.4		
pig Dns-AT_{69}PLA	355	0.27		
pig NT_{123}PLA[a]		nd	6.90	nd
pig AT_{123}PLA[a]		5.5		
pig Dns-AT_{123}PLA[a]	355	0.8		
ox phospholipase A_2 (PLA)		>13		
ox NT_{69}PLA	458	5.0	6.50	6.40
ox Dns-AT_{69}PLA	355	1.0		

[a] Not determined (nd) because of the contamination with nitrotyrosine-69 phospholipase; in contrast to the nitrotyrosyl-123 derivative, the aminotyrosyl and dansylaminotyrosyl phospholipases have been obtained in pure form (see preceding paper in this issue).

binding which returns to about normal upon conversion into the aminotyrosyl derivative. The difference spectrum of equine NT_{69}PLA resembles that of porcine NT_{69}PLA, although some slight differences can be seen (Table I). The difference spectrum of porcine NT_{123}PLA in the presence of the phospholipid (not shown) is dominated by the normal tryptophan signals at 291 nm and a slight difference absorption band at 455 nm. This nitrotyrosine contribution can be totally ascribed to the contamination with NT_{69}PLA [Meyer et al. (1979), preceding paper]. With respect to the nitrotyrosine perturbation, horse NT_{19}PLA shows a difference spectrum (Figure 1E) resembling that of Figure 1C. The blue shift of 13 nm of the minimum (from 431 to 418 nm) indicates a hydrophobic perturbation of this residue and is accompanied by a considerable increase of the p*K* from 7.4 to 8.1. The K_D value given in Table I suggests that the affinity toward micelles is greatly enhanced. However, for the aminotyrosine derivative the affinity is somewhat decreased.

Bovine phospholipase A_2 is exceptional because at pH 6 micellar binding is extremely weak (P. S. de Araujo and G. H. de Haas, unpublished experiments). However, its nitrotyrosyl-69 derivative, like the other nitrotyrosine-69 enzymes, shows a noticeable increase in micellar binding (Table I). Neither the nitrated proenzymes nor the model compound displays any difference absorbance signals upon addition of micellar phospholipid.

The dansyl group, covalently attached to a specific aminotyrosine, is an extremely sensitive probe for microenvironmental hydrophobic changes (Kenner & Neurath, 1971; Chen, 1968; Chen & Kernohan, 1967). This probe can be monitored by fluorescence spectroscopy as well as by ultraviolet difference spectroscopy.

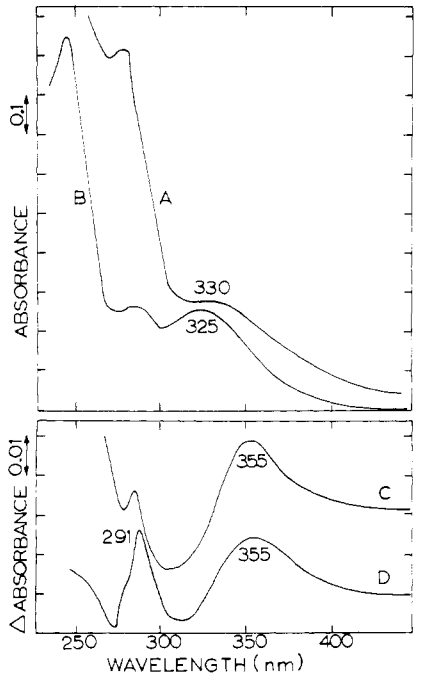


FIGURE 2: Ultraviolet (difference) absorbance spectra produced by *N*-acetyl-3-dansylaminotyrosine ethyl ester and horse dansylaminotyrosine-69 phospholipase A_2 upon interaction with *n*-hexadecylphosphocholine. The following are the experimental conditions. Absorbance spectra of the protein (45 μM; curve A) and of the model compound (55 μM; curve B) were measured in 0.1 M NaCl and 50 mM sodium acetate, pH 6.0. Difference absorbance spectra of the model compound (55 μM; curve C) and of the protein (45 μM; curve D) were recorded in the presence of 5 mM *n*-hexadecylphosphocholine by using the same buffer as described under Methods.

Ultraviolet difference spectroscopy was used to quantify micellar binding of the dansylaminotyrosine phospholipases. Figure 2A shows the absorption spectrum of horse dansylaminotyrosine-69 phospholipase A_2. The absorption spectrum of *N*-acetyl-3-dansylaminotyrosine ethyl ester, used as a model compound, is shown in Figure 2B. In both spectra the dansyl absorption band at 325 and 330 nm, respectively, can be clearly distinguished. Addition of *n*-hexadecylphosphocholine to the model compound produces a difference spectrum as shown in Figure 2C. The large red shift of the maximum suggests that the probe moves into the hydrophobic environment of the micelles. The difference spectrum produced by Dns-AT_{69}PLA upon micellar binding is shown in Figure 2D. Besides the tryptophan perturbation at 291 nm, a similar red shift of the dansylaminotyrosine difference absorption can be observed, strongly suggesting that this residue also moves into the micellar substrate. The other dansylaminotyrosine enzymes produce similar spectra. K_D values have been determined as already described for the nitrotyrosine enzymes (Table I). As can be seen, micellar binding of the Dns-AT_{69}PLA, and in particular horse Dns-At_{19}PLA, is tremendously improved. As a control, the dansylaminotyrosine proenzymes have also been titrated. No difference absorption signals were obtained, indicating that these modified zymogens do not bind to the micellar substrate analogue.

Fluorescence Spectroscopy. Changes in fluorescence intensity and in the position of the emission maximum of the dansyl moiety are parameters frequently used to probe its microenvironment in proteins (Kenner & Neurath, 1971). Changes in these parameters in the presence of substrate reflect the accessibility of the probe toward hydrophobic interactions. These spectral changes have been investigated for some dansyl amino acids in the presence of *n*-hexadecylphosphocholine

Table II: Fluorescence Properties of Some Dansyl Amino Acids (20 μM) upon Interaction with *n*-Hexadecylphosphocholine in 0.1 M NaCl and 50 mM Sodium Acetate at pH 6.0 and 25 °C[a]

compd	% increase of dansyl fluoresence[b]	wavelength (nm) of the emission max	
		without lipid	10 mM lipid
N-acetyl-3-danyslaminotyrosine ethyl ester	670	546	520
dansylglycine	210	545	524
dansyltryptophan	620	545	515
dansylphenylalanine	420	545	516
ϵ-*N*-dansyllysine	500	542	515
dansylglutamic acid	50	545	532
dansyl-OH	25	496	488

[a] Excitation was performed at 330 nm. [b] Percent increase as compared to the fluorescence in the absence of lipid.

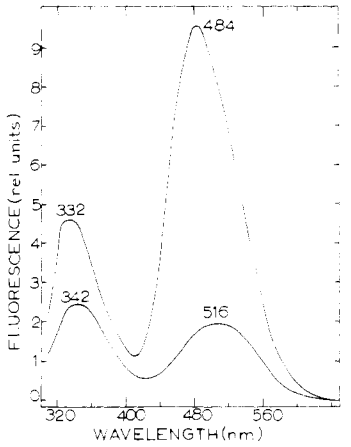


FIGURE 3: Tryptophan and dansyl emission spectra of horse dansylaminotyrosine-69 phospholpase A_2 produced in the absence and presence of *n*-hexadecylphosphocholine. The following are the experimental conditions. The protein solution (15 μM), in 0.1 M NaCl and 50 mM sodium acetate, pH 6.0, was excitated at 295 nm. Excitation and emission slit widths were set at 5 nm. The lower and upper curves are in the absence and presence of 5 mM *n*-hexadecylphosphocholine, respectively.

(Table II). Two extreme cases were observed: the model compound *N*-acetyl-3-dansylaminotyrosine ethyl ester shows a large increase in fluorescence (670%) and a concomitant blue shift of the emission maximum (26 nm), whereas dansylglutamic acid and dansyl OH show only an increase in fluorescence of 50 and 25%, respectively, and much smaller shifts. From these data it is obvious that dansylaminotyrosine is an excellent fluorescent probe for following hydrophobic changes due to micellar binding. Fluorescence of the dansylaminotyrosine enzymes can be monitored in two ways. Excitation at 295 nm causes tryptophan as well as dansyl fluorescence (Conrad & Brand, 1968). A typical example is shown in Figure 3 for horse Dns-AT_{19}PLA in the absence and presence of phospholipid. Tryptophan fluorescence increased by 90%, coinciding with a blue shift of 10 nm (from 342 to 332 nm), whereas the dansyl fluorescence increased by 420% with a blue shift of 32 nm (from 516 to 434 nm). In order to observe only dansyl emission, we used excitation at 330 nm. At this wavelength there is no energy transfer from tryptophan to the dansyl group (Kenner & Neurath, 1971). A typical example upon titration with *n*-hexadecylphosphocholine is shown in Figure 4 for horse Dns-AT_{69}PLA and Dns-AT_{19}PLA. It is obvious that the increase of fluorescence and blue shift of the emission maximum are much more pronounced for

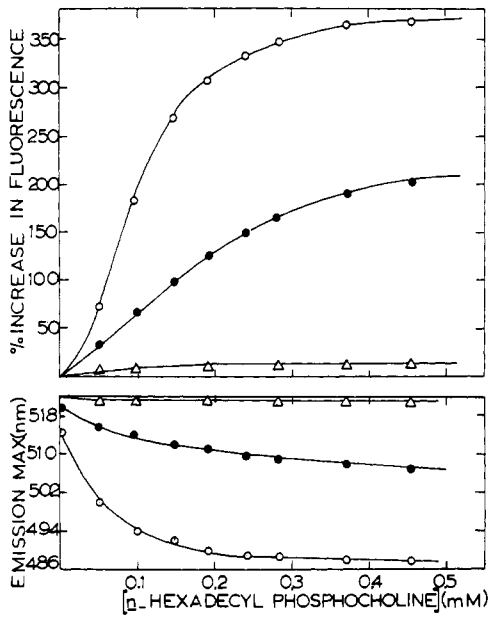


FIGURE 4: Percent increase in dansyl fluorescence and shift in the maximal emission wavelength produced upon interaction of horse dansylaminotyrosine-69 phospholipase (●, 15 μM), horse dansylaminotyrosine-19 phospholipase (○, 15 μM), and horse dansylaminotyrosine-69 (pro)phospholipase (△, 15 μM) with *n*-hexadecylphosphocholine. The following are the experimental conditions. The protein solution in 0.1 M NaCl and 50 mM sodium acetate, pH 6.0, was excitated at 330 nm. Excitation and emission slit widths were set at 5 nm.

Table III: Some Fluorescence Properties of Horse, Pig, and Ox Dansylaminotyrosine (Pro)phospholipases A_2 upon Interaction with *n*-Hexadecylphosphocholine (10 mM) at pH 6.0 and 25 °C[a]

enzyme derivative	% increase of dansyl fluorescence	wavelength (nm) of the emission max: without lipid	wavelength (nm) of the emission max: 10 mM lipid
horse Dns-AT_{19}PLA	400	515	488
horse Dns-AT_{69}PLA	240	520	506
horse Dns-AT_{69}proPLA	15	520	520
pig Dns-AT_{69}PLA	310	516	495
pig Dns-AT_{69}proPLA	20	516	516
pig Dns-AT_{123}PLA	130	516	495
pig Dns-AT_{123}proPLA	20	516	516
ox Dns-AT_{69}PLA	90	516	504
ox Dns-AT_{69}proPLA	5	516	516

[a] Excitation was performed at 330 nm.

Dns-AT_{19}PLA, suggesting that the dansylaminotyrosine residue 19 penetrates further into the micelle than the dansylaminotyrosine-69 in Dns-AT_{69}PLA (compare the model compound). The observed changes in both fluorescent parameters are characteristic of the dansylated active enzymes. The corresponding zymogens show only a minor increase in fluorescence and no shift of the emission maximum (Figure 4). The data for all dansylated (pro)phospholipases are summarized in Table III.

Monomer Phospholipid Binding. Interaction of porcine phospholipase A_2 with monomer substrate analogues (*n*-dodecylphosphocholine) below the critical micellar concentration (1.1 mM) gives rise to difference absorption spectra dominated by tyrosine perturbations at 282 and 288 nm, as can be seen from Figure 5B (van Dam-Mieras et al., 1975; Pieterson et al., 1974b). It was of particular interest to investigate the contribution of the modified tyrosines to the observed difference spectrum and hence to study the possible role of these residues in monomer phospholipid binding. Titration of the (modified) phospholipases with *n*-dodecylphosphocholine up to the cmc was performed at pH 6.0 and 8.0. Figure 5A shows the difference spectrum of porcine NT_{69}PLA in the presence of 1.0 mM phospholipid at pH 6.0. The spectrum is dominated by a nitrotyrosine perturbation spectrum resembling that of Figure 1C. The large blue shift of the minimum (from 431 to 397 nm) indicates a very hydrophobic perturbation of this nitrotyrosine. The spectra belonging to the lower lipid concentrations have been omitted from these figures, but they have been used in the calculation of the K_D value as described above. Table IV summarizes the data for some of the modified phospholipases. As can be seen, phospholipase and NT_{69}PLA bind monomer phospholipid with equal affinities at pH 6.0. However, at pH 8.0, phospholipid binding to NT_{69}PLA is greatly impaired ($K_D > 5$ mM, compared to the native enzyme, in which $K_D = 0.7$ mM). These results strongly suggest that the ionized nitrotyrosine at pH 8.0 causes this decrease in binding. Additional evidence was obtained from the titration of AT_{69}PLA with monomer phospholipids. The difference spectrum in the presence of 1.0 mM phospholipid (Figure 5C) is dominated by a hydrophobic perturbation of aminotyrosine

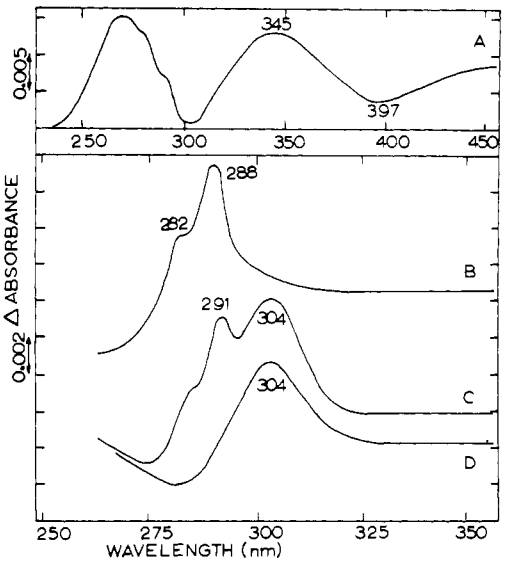


FIGURE 5: Difference absorption spectra produced by the interaction of pig nitrotyrosine-69 phospholipase (A), pig phospholipase (B), and pig aminotyrosine-69 phospholipase (C) with *n*-dodecylphosphocholine below the critical micellar concentration. The following are the experimental conditions. Protein concentrations were 40 μM in 0.1 M NaCl and 50 mM sodium acetate, pH 6.0. Phospholipid was added up to 1.0 mM as described under Methods. Solvent perturbation of *N*-acetyl-3-aminotyrosine ethyl ester (curve D) was measured in 0.2 M Pipes, pH 6.0; 50% dioxane (v/v) was present in the sample compartment and in the buffer solution of the reference compartment.

Table IV: Dissociation Constants of *n*-Dodecylphosphocholine with Porcine Nitrotyrosine and Aminotyrosine Phospholipase A_2 as Obtained from Ultraviolet Difference Spectroscopy at 25 °C

	K_D (mM) ± 10%, pH 6.0	K_D (mM) ± 10%, pH 8.0
pig phospholipase A_2	0.3	0.7
pig NT_{69}PLA	0.3	>5[a]
pig AT_{69}PLA	0.5	1.2
pig NT_{123}PLA	0.3	0.7

[a] Due to the limited concentration of *n*-dodecylphosphocholine that could be used (up to the cmc = 1.1 mM), a precise K_D value could not be obtained, but this value is certainly not less than 5 mM.

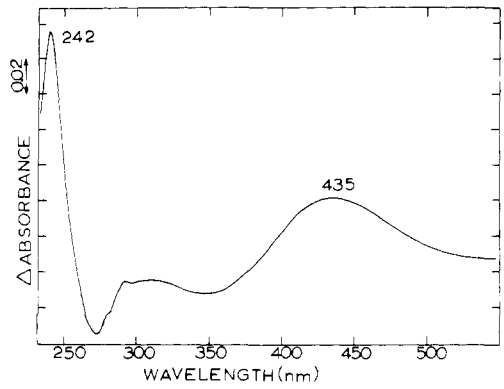


FIGURE 6: Difference absorption spectrum produced by the interaction of horse nitrotyrosine-69 phospholipase A_2 with Ca^{2+}. The following are the experimental conditions. Protein solution (40 μM) in 0.1 M NaCl and 50 mM sodium acetate, pH 6.0. $CaCl_2$ was added up to 6 mM as described under Methods.

(peak at 304 nm). A similar perturbation is observed with *N*-acetyl-3-aminotyrosine ethyl ester in 50% dioxane at pH 6.0 (Figure 5D). Moreover, it is also clear from Figure 5C that some other tyrosine residues are perturbated because of the superposition of a normal tyrosine perturbation, as reflected by the peak at 291 nm. The K_D values calculated from the amplitudes at 304 nm are 0.5 and 1.2 mM at pH 6.0 and 8.0, respectively. This provides evidence that the negative charge of nitrotyrosine-69 at pH 8.0 is the predominant factor responsible for the impairment of monomer phospholipid binding. Porcine $NT_{123}PLA$ exhibits a normal tyrosine difference spectrum, similar to that shown in Figure 5B. The K_D values (0.3 and 0.7 mM at pH 6.0 and 8.0, respectively) show that this tyrosine is not perturbed by monomer phospholipid binding.

Titration of horse $NT_{69}PLA$ and horse $NT_{19}PLA$ as well as of bovine $NT_{69}PLA$ with monomers of *n*-dodecylphosphocholine produced nitrotyrosine difference spectra closely resembling that of Figure 5A. Apparently these latter tyrosines are also perturbed upon monomer binding. No K_D values could be determined due to the low affinity of these enzymes for monomers and the limited concentration range set by the cmc of the lipid.

Ca^{2+} Binding of the Nitrated (Pro)phospholipases. As already discussed by Pieterson et al. (1974a), addition of Ca^{2+} ions to (pro)phospholipase A_2 produces a difference spectrum characterized by a large peak at 242 nm and very small peaks around 288 nm. These peaks have been attributed to perturbations of histidine and one or more tyrosine side chain(s). Alkylation of histidine-48 (Volwerk et al., 1974) causes a complete loss of both Ca^{2+} and monomer phospholipid binding as judged by several techniques, including ultraviolet difference spectroscopy. It was of interest, therefore, to examine the influence of Ca^{2+} binding on the environment of the nitrotyrosine in the nitrated (pro)enzymes. Figure 6 shows a typical difference spectrum of horse $NT_{69}PLA$ in the presence of 6 mM Ca^{2+} ions. The spectrum is characterized by the expected peak at 242 nm and, in addition, a large nitrotyrosine perturbation. The spectrum closely resembles that of Figure 1B, suggesting that a predominant charge effect occurs. The amplitudes at 242 and 435 nm have been used to determine the $K_{Ca^{2+}}$ values (Table V). Spectrophotometric pH titration revealed a considerable decrease of the p*K* of nitrotyrosine-69 in the presence of Ca^{2+} (Table V). Porcine NT_{69}(pro)PLA and bovine NT_{69}(pro)PLA produce similar difference spectra and show a decrease of the p*K* values upon Ca^{2+} binding (Table V). Porcine $NT_{123}PLA$ and horse $NT_{19}PLA$ do not produce any nitrotyrosine difference spectra, proving that these tyrosines are not perturbed by Ca^{2+} binding.

Table V: Some Properties of Horse, Pig, and Ox (Pro)phospholipases and Their Nitrated Derivatives upon the Interaction of Ca^{2+} as Determined from Ultraviolet Difference Spectroscopy at pH 6.0 and Spectrophotometric pH Titration at 25 °C[a]

enzyme derivative	λ_{max} of the difference spectrum (nm)	p*K* of nitrotyrosine		
		$K_{Ca^{2+}}$ (mM) ± 10%	without Ca^{2+}	30 mM Ca^{2+}
horse phospholipase A_2		1.1		
horse $NT_{19}PLA$	–	1.1	7.4	7.4
horse $NT_{69}PLA$	432	0.9	7.2	6.7
horse proPLA		3.3		
horse NT_{69}proPLA	435	2.5	6.9	6.5
pig phospholipase A_2		2.5		
pig $NT_{69}PLA$	435	2.5	7.1	6.6
pig proPLA		2.9		
pig NT_{69}proPLA	433	2.9	nd	nd
pig $NT_{123}PLA$	–	2.5	nd	nd
ox phospholipase A_2		8.0		
ox $NT_{69}PLA$	434	11.0	6.5	5.8
ox proPLA		8.0		
ox NT_{69}proPLA	434	10.7	nd	nd

[a] nd stands for not determined.

Obviously, perturbation of nitrotyrosine-69 by Ca^{2+} does not affect the Ca^{2+} binding as can be concluded from Table V. In order to rule out effects caused by unspecific Ca^{2+} binding we titrated the nitrotyrosine-69 enzymes, modified with 1-bromo-2-octanone, with Ca^{2+}. These modified enzymes do not show any difference absorption signals.

Kinetic Experiments. Kinetic studies of phospholipase A_2 acting on monomolecular surface films have been shown to provide valuable information on the influence of the phospholipid–water interface on the turnover of the enzyme (Verger et al., 1973). However, the two-dimensional state of the substrate prevents treatment of the data by the classical Michaelis–Menten theory, and it has been proposed (Verger et al., 1973) that hydrolysis of the substrate molecule in the monolayer is preceded by two successive equilibria:

$$E \leftrightharpoons E^* + S \leftrightharpoons E^*S \rightarrow E^* + P$$

The first step involves the reversible penetration of the enzyme E from the aqueous bulk phase into the phospholipid monolayer: $E \rightleftarrows E^*$. In the interface the enzyme molecule E*, having a conformation slightly different from that of the soluble enzyme E, reversibly forms a two-dimensional enzyme–substrate complex E*S, which subsequently decomposes into the product P and E*. It has been shown that the packing density of the substrate molecules at the air–water interface has a dramatic effect on the penetration rate of the enzyme in the film and that above a certain well-defined surface pressure the enzyme is no longer able to penetrate the monolayer. At this point hydrolysis abruptly stops.

For the pancreatic phospholipases, A_2, which are characterized by a rather low penetrating capacity (Verger et al., 1973), the establishment of the equilibrium $E \leftrightharpoons E^*$ is usually the rate-limiting step of the overall enzymatic process. A steady-state hydrolysis rate is found only after a certain induction time τ. Therefore, the dependence of the induction time τ on the surface pressure π of the monolayer is a sensitive parameter which can be used to quantify the penetrating power of a particular enzyme.

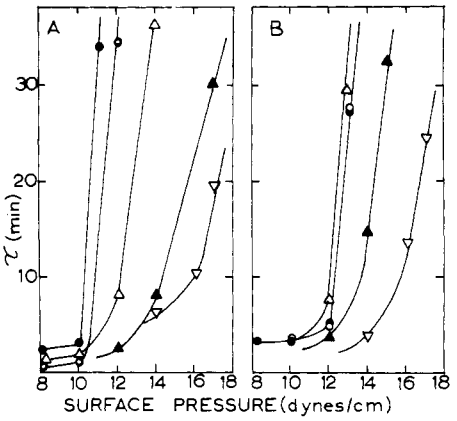


FIGURE 7: Influence of the surface pressure (π) of a 1,2-didecanoyl-*sn*-glycero-3-phosphocholine monolayer on the induction time (τ) of several modified phospholipases. (A) (●) Horse phospholipase; (○) horse nitrotyrosine-69 phospholipase; (△) horse nitrotyrosine-19 phospholipase; (▲) horse dansylaminotyrosine-69 phospholipase; (▽) horse dansylaminotyrosine-19 phospholipase. (B) (●) Pig phospholipase; (▲) pig nitrotyrosine-69 phospholipase; (○) pig nitrotyrosine-123 phospholipase; (▽) pig dansylaminotyrosine-69 phospholipase; (△) pig dansylaminotyrosine-123 phospholipase. The following are the experimental conditions: 10 mM Tris–sodium acetate, 0.1 M NaCl, and 20 mM $CaCl_2$, pH 6.0, and 25 °C.

Figure 7 shows the τ–π profiles for the various modified phospholipases from horse (7A) and pig (7B) at pH 6.0. It is clear that nitration of tyrosine-69 and -19 results in enzyme species with a somewhat higher penetrating capacity, whereas nitration of tyrosine-123 in the porcine enzyme does not change the lipid binding site.

The attachment of the hydrophobic dansyl group to tyrosine-69 and -19 gives rise to a further enhancement of the penetrating power of both enzymes. A similar behavior was observed for the nitrated and dansylated bovine enzyme (not shown). As can be seen, the dansyl group of tyrosine-123 in the porcine enzyme has hardly any influence on its penetrating power. At a fixed surface pressure, the induction time τ as a function of pH provides information on the effect of ionizing groups on the penetrating power of the enzyme. Figure 8 gives the τ–pH profiles for the several modified enzymes from horse (8A) and pig (8B) at surface pressures of 10 and 12 dyn/cm, respectively. Between pH 6.0 and pH 10, at this rather low surface pressure, the native horse phospholipase penetrates the film quickly. The introduction of a nitro group on tyrosine-69, however, greatly diminishes the penetrating capacity above pH 8.5, probably because of the negative charge on this tyrosine due to its rather low pK of 6.9 (Table I).

A similar picture is found for the porcine enzyme (Figure 8B). The native enzyme loses its penetration capacity above pH 9.0 due to the deprotonation of the α-NH_3^+ group of the N-terminal Ala_1. However, the introduction of a nitro group on tyrosine-69, which lowers the pK of this residue to 6.5 (Table I), rapidly destroys the lipid binding properties at pH values above 7. Upon conversion of the nitro group on tyrosine-69 into an amino group, the normal pK of tyrosine is restored, and the penetrating capacity of the aminotyrosine-69 enzyme strongly resembles that of the native phospholipase (not shown).

Although nitrotyrosine-123 also has a pK value less than 7, the introduction of a negative charge on this residue has hardly any influence on the penetration capacity of the enzyme.

Discussion

Interaction of Modified Phospholipases with Micellar and Monolayer Phospholipid–Water Interfaces. Ultraviolet difference spectra of the nitrated phospholipase A_2 complexes

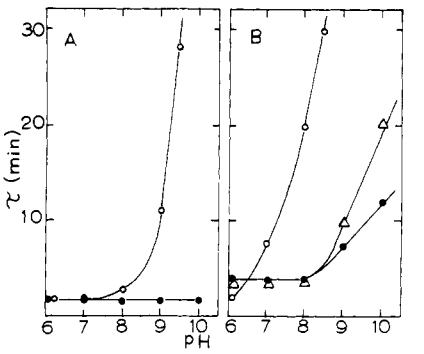


FIGURE 8: Influence of the pH of the subphase on the induction time (τ) during hydrolysis of a 1,2-didecanoyl-*sn*-glycero-3-phosphocholine film. (A) (●) Horse phospholipase; (○) horse nitrotyrosine-69 phospholipase. (B) (●) Pig phospholipase; (○) pig nitrotyrosine-69 phospholipase; (△) pig nitrotyrosine-123 phospholipase. The following are the experimental conditions: 10 mM Tris–sodium acetate, 0.1 M NaCl, and 20 mM $CaCl_2$; surface pressure of the monolayer was 10 dyn/cm for the horse enzymes (A) and 12 dyn/cm for the pig enzymes (B).

with micellar substrate analogues indicated changes in the environment of the invariant tyrosine-69. Two effects have been observed: a decrease of the pK of nitrotyrosine and a concomitant large red shift of the difference absorption maximum.

The first effect is thought to be caused by the approach of a positively charged group. From the three-dimensional structure of bovine phospholipase A_2 (Dijkstra et al., 1978), it is seen that such a charged group might be the invariant lysine-56. On the other hand, the positively charged choline group of the phospholipid could also be responsible.

The second effect must be due to the more hydrophobic environment of tyrosine-69, probably caused by a fatty acid chain of the bound phospholipid.

It has to be remarked, however, that the behavior of nitrotyrosine-69 upon hydrophobic perturbation is unusual: a decrease of the pK is observed instead of the expected increase (Donovan, 1970). In contrast, the perturbation spectrum and spectrophotometric pH titration of horse $NT_{19}PLA$ upon micellar binding show that this residue behaves as expected: an increase of the pK and a concomitant hydrophobic perturbation. As we do not know the exact position of this tyrosine (bovine phospholipase has leucine in this position), it is difficult to predict the influence of adjacent amino acid residues. However, it is highly probable that (nitro)tyrosine-19 interdigitates with the fatty acid chains of the micellar phospholipid. It is reasonable to assume that the presence of the nitro groups on tyrosine-69 and -19 is responsible for the improved micellar binding (see Table I). This conclusion is supported by the fact that reductive transformation of nitrotyrosine into aminotyrosine derivatives, which can be expected to increase the polar character of tyrosine, roughly restores the affinity of the enzymes for lipid micelles to values similar to those of the native enzymes.

As would be expected, the introduction of an apolar dansyl side chain on the aminotyrosine-69 and -19 strongly improves the affinity of the enzyme for lipid–water interfaces. This improved affinity indicates that both residues, and in particular tyrosine 19, are directly involved in the hydrophobic interaction with the apolar fatty acid chain of the phospholipid. The data obtained from fluorescence spectroscopy confirm these findings. From the increase of fluorescence and blue shift of the emission maximum, it can be concluded that the dansylaminotyrosine-19 moiety probably moves into the micelle completely, whereas the interaction of the dansylamino-

tyrosine-69 group with the micelles is somewhat less strong. One has to be careful in the interpretation of the results of micellar binding on porcine Dns-AT_{123}PLA. In our opinion, tyrosine-123 is not a direct part of the micellar binding site but might be localized in close proximity to it. Once the bulky dansyl group is attached to tyrosine-123, this residue might reach the micelles in some way, thus explaining the slight increase of fluorescence and improvement of micellar binding.

In contrast to the dansylated enzymes, the dansylated proenzymes neither show enhancement nor shift the emission maximum of their fluorescence upon addition of micelles. These observations, therefore, provide strong evidence for the specific binding of the active enzymes to the micellar lipid–water interfaces.

Micellar binding to the (modified) phospholipases, as discussed so far, reflects equilibrium conditions. Pre-steady-state data obtained from monolayer kinetics provide additional information about the tyrosine residues during the interaction of phospholipase A_2 with lipid–water interfaces. The dependence of the most important parameter, the induction time τ, on the surface pressure of the monolayer reflects the ability of the enzyme to penetrate into the lipid film. Although one might expect that this "dynamic" parameter is not necessarily related to the "static" K_D values as obtained by ultraviolet difference spectroscopy, a remarkable correlation is observed. In particular, the considerably stronger penetration powers of horse NT_{19}PLA and its dansyl derivative are clear examples. However, the nitrotyrosyl-69 enzymes and their dansyl derivatives show this correlation to a lesser extent. Moreover, the monolayer technique revealed that modification of tyrosine-123 in the porcine enzyme does not alter the penetration capacity of this derivative compared to that of the native enzyme. Measurement of induction time as a function of pH elucidates the effect of charge upon the penetrating power of the enzymes. This has been demonstrated for the nitrotyrosine-69 phospholipases. Without any doubt the negative charge of these amino acid residues at pH values above 8 hampers the penetration of these enzymes into the monolayer. This idea is strengthened by the fact that the aminotyrosyl and dansylaminotyrosyl derivatives behave like the native enzymes (not shown). In addition, porcine NT_{123}PLA behaves like the native enzyme. Obviously, the negative charge of this nitrotyrosine at basic pH values does not influence penetration capacity. Unfortunately, the τ–pH profile of horse NT_{19}PLA (not shown) could not be interpreted accurately because comparison with the native enzyme using identical surface pressure was not possible.

Considering all the data together, it can be concluded that the actual micellar binding site of pancreatic phospholipase A_2 involves the NH_2-terminal region (van Dam-Mieras et al., 1975) and in addition at least tyrosine-69 and tyrosine-19 (in horse phospholipase). Although Tyr_{19} is not an invariant residue, it can be argued that the amino acids comprising the micellar binding site may vary to a certain extent without lethal consequences (Slotboom & de Haas, 1975). Such a situation would not be possible at the catalytic site.

Monomer Phospholipid Binding. Monomer phospholipid binding experiments with porcine NT_{69}PLA and AT_{69}PLA, using *n*-dodecylphosphocholine as the substrate analogue and assaying with ultraviolet difference spectroscopy, revealed that binding of this substrate analogue to the nitrotyrosine enzyme at pH 6.0 is similar to that of the native enzyme, whereas at pH 8.0 binding is greatly impaired ($K_D > 5$ mM; native enzyme, $K_D = 0.7$ mM). Due to the limited possible concentration range (up to the critical micellar concentration of 1.1 mM) of the monomeric phospholipid, an accurate determination of K_D is rather difficult. However, K_D is certainly not less than 5 mM. Obviously, the negative charge of the ionized nitrotyrosine causes the decrease of binding. This is because AT_{69}PLA, the aminotyrosine of which is not ionized at pH 8.0, only shows a slight decrease of binding. In addition, the fact that nitrotyrosine-69 undergoes a rather strong hydrophobic perturbation upon monomer phospholipid binding, as shown by ultraviolet difference spectroscopy, provides sufficient evidence to conclude that tyrosine-69 is directly involved in monomer phospholipid binding. The knowledge of the three-dimensional structure of bovine phospholipase A_2, together with experimental data already discussed, leads to the following hypothesis. Tyrosine-69 may provide a phenolic hydrogen atom in order to form a hydrogen bridge with one of the oxygen atoms of the phosphate group of the bound phospholipid. Once this tyrosine is nitrated, the negatively charged phosphate group of the phospholipid may be repelled by the negatively charged phenolic group of this nitrotyrosine at pH 8.0. This effect is diminished when nitrotyrosine is not ionized (at pH 6.0) or upon conversion of this amino acid into aminotyrosine. The ultraviolet difference spectrum of AT_{69}PLA in the presence of phospholipid (Figure 5C) suggests the perturbation of an additional tyrosine. A possible candidate is tyrosine-52, because the three-dimensional structure of bovine phospholipase shows that this residue is in very close proximity to both tyrosine-69 and the active site histidine-48.

Some remarks have to be made with regard to the monomer phospholipid binding of horse NT_{19}PLA. The difference spectrum suggests that this tyrosine is also considerably perturbed, although binding to the phospholipid could not be quantified. It is highly probable that the observed hydrophobic perturbation is also caused by a fatty acid chain of the bound phospholipid.

All data discussed so far lead to the conclusion that the micellar binding site of phospholipase A_2 partially overlaps the monomer phospholipid binding site. Moreover, immunological studies on pancreatic phospholipases (Meyer et al., 1978) support this point of view.

Ca^{2+} Binding. It was found that binding of the essential cofactor Ca^{2+} to the tyrosine-69 nitrated (pro)phospholipases gave rise to a considerable charge effect on this tyrosine, whereas porcine NT_{123}(pro)PLA and horse NT_{19}PLA did not show any nitrotyrosine perturbations in the presence of Ca^{2+} ions. As has been suggested by Pieterson et al. (1974a), the difference absorbance signal at 242 nm, generated by Ca^{2+} binding to phospholipase, can be attributed to a perturbation of one or more tyrosine and one histidine side chain. Therefore, it is reasonable to assume that one of these tyrosine residues can be assigned to tyrosine-69. The large decrease in the p*K* of nitrotyrosine-69 in the presence of Ca^{2+} indicates the proximity of a positively charged group. This effect is probably not caused by the presence of the Ca^{2+} ion itself, because the three-dimensional structure of the enzyme shows that the distance between the Ca^{2+} binding site and tyrosine-69 is approximately 10 Å. A more attractive candidate for the observed charge effect is the side chain of lysine-56 which is located within hydrogen-bond distance from tyrosine-69.

Acknowledgments

The authors are much indebted to Dr. E. Burnell (University of British Columbia, Vancouver, Canada) for carefully proofreading the manuscript.

References

Chen, R. F. (1968) *Arch. Biochem. Biophys. 128*, 163–175.

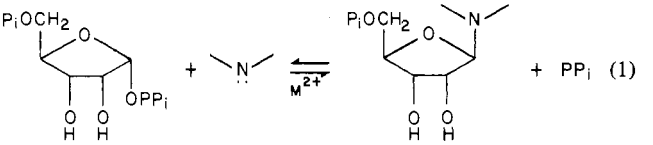
Chen, R. F., & Kernohan, J. C. (1967) *J. Biol. Chem. 242*, 5813–5823.
Conrad, R. H., & Brand, L. (1968) *Biochemistry 7*, 777–787.
Cuatrecasas, P., Fuchs, S., & Anfinsen, C. B. (1968) *J. Biol. Chem. 243*, 4787–4798.
Cubero Robles, E., & van den Berg, D. (1969) *Biochim. Biophys. Acta 187*, 520–526.
Dijkstra, B. W., Drenth, J., Kalk, K. H., & Vandermaelen, P. J. (1978) *J. Mol. Biol. 124*, 53–60.
Donovan, J. W. (1970) in *Physical Principles and Techniques of Protein Chemistry* (Leach, S. J., Ed.) Part A, pp 101–169, Academic Press, New York.
Furth, A. J., & Hope, D. B. (1969) *Biochem. J. 116*, 545–553.
Kenner, R. A. (1971) *Biochemistry 10*, 545–550.
Kenner, R. A., & Neurath, H. (1971) *Biochemistry 10*, 551–557.
Lineweaver, H. L., & Burk, D. (1934) *J. Am. Chem. Soc. 56*, 658–666.
Mangold, H. K. (1973) *Chem. Phys. Lipids 11*, 244–253.
Meyer, H., Meddens, M., Dijkman, R., Slotboom, A. J., & de Haas, G. H. (1978) *J. Biol. Chem. 253*, 8564–8569.
Meyer, H., Verhoef, H., Hendriks, F. F. A., Slotboom, A. J., & de Haas, G. H. (1979) *Biochemistry* (preceding paper in this issue).
Pieterson, W. A., Volwerk, J. J., & de Haas, G. H. (1974a) *Biochemistry 13*, 1439–1445.
Pieterson, W. A., Vidal, J. C., Volwerk, J. J., & de Haas, G. H. (1974b) *Biochemistry 13*, 1455–1460.
Slotboom, A. J., & de Haas, G. H. (1975) *Biochemistry 14*, 5394–5399.
Sokolovsky, M., Riordan, J. F., & Vallee, B. L. (1966) *Biochemistry 5*, 3582–3589.
Sokolovsky, M., Riordan, J. F., & Vallee, B. L. (1967) *Biochem. Biophys. Res. Commun. 27*, 20–25.
van Dam-Mieras, M. C. E., Slotboom, A. J., Pieterson, W. A., & de Haas, G. H. (1975) *Biochemistry 14*, 5387–5394.
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.
Visser, L., Sigman, D. S., & Blout, E. R. (1971) *Biochemistry 10*, 735–742.
Volwerk, J. J., Pieterson, W. A., & de Haas, G. H. (1974) *Biochemistry 13*, 1446–1454.

Divalent Metal Ion Activation of the Yeast Orotate Phosphoribosyltransferase Catalyzed Reaction†

Jacob Victor,‡ Anthony Leo-Mensah, and Donald L. Sloan*

ABSTRACT: An analysis of the divalent metal ion activation of the orotate phosphoribosyltransferase catalyzed reaction has been performed using homogeneous preparations of this enzyme from yeast. Electron paramagnetic resonance (EPR), UV spectroscopy, and water proton magnetic relaxation rate (PRR) measurements have been utilized to characterize dissociation constants ($\bar{K}_0$) for the formation of binary complexes between Mn^{2+} and orotate ($\bar{K}_0$ = 500 μM), phosphoribosyl pyrophosphate (P-Rib-PP; $\bar{K}_0$ = 30 μM), PP_i ($\bar{K}_0$ = 12 μM), and OMP ($\bar{K}_0$ = 200 μM). EPR and PRR evidence has been collected for the formation of a binary Mn^{2+}–enzyme complex (maximal stoichiometry 4:1). The shape of the binding isotherm which defines this complex formation is sigmoidal, suggesting cooperation between Mn^{2+} binding sites. Kinetic analysis of the activation of the phosphoribosyl transfer reaction revealed a biphasic nature for Mg^{2+} and Mn^{2+} activation. These results are interpreted in terms of an enzyme–metal ion complex and a metal-free enzyme, both of which catalyze this reaction but at different rates. Although Co^{2+} and Ca^{2+} do not activate the phosphoribosyl transfer, Co^{2+} inhibits Mg^{2+} activation (perhaps as a result of the formation of a Co^{2+}–orotate complex, K_0 = 40 μM). Previous kinetic studies [Victor, J., Greenberg, L. B., & Sloan, D. L. (1979) *J. Biol. Chem. 254*, 2647–2655] revealed that this phosphoribosyl transfer proceeds through the use of a ping-pong kinetic mechanism in the presence of optimal Mg^{2+} concentrations and that both half reactions, as defined by radioactive exchange between substrate/product pairs, require Mg^{2+}. The present results suggest that the M^{2+}–orotate phosphoribosyltransferase complex may be the enzyme form which catalyzes the reaction via this mechanism.

Divalent metal ion activators such as Mg^{2+} or Mn^{2+} are required for all phosphoribosyltransferase-catalyzed reactions in which β-glycosidic linkages are formed between the C-1 position of phosphoribosyl α-1-pyrophosphate[1] (P-Rib-PP) and the appropriate nitrogenous second substrates (eq 1).



(1)

The nature of this activation, however, is not clearly understood. It has been suggested or assumed that the monomagnesium or dimagnesium salts of P-Rib-PP are the true substrates for reactions catalyzed by adenine phosphoribosyltransferase from *Escherichia coli* (Berlin, 1969; Hochstadt-Ozer & Stadtman, 1971), by hypoxanthine–guanine

† From the Department of Chemistry, City College of the City University of New York, New York, New York 10031. *Received January 10, 1979.* This research was supported by Grant BC-268 from the American Cancer Society, by Grant AM-20183 from the National Institutes of Health, and by awards from the Research Corporation and the Research Foundation of CUNY. An abstract of this work was presented at the Conference on Methods for Determining Metal Ion Environments in Proteins, Jan 1979, New Mexico State University, Las Cruces, NM.

‡ Present address: Roche Institute of Molecular Biology, Nutley, NJ 07110.

[1] Abbreviations used: P-Rib-PP, 5-phosphoribosyl α-1-pyrophosphate; OMP, orotidine 5′-phosphate; PP_i, inorganic pyrophosphate; PRR, water proton magnetic relaxation rate; EPR, electron paramagnetic resonance.

0006-2960/79/0418-3597$01.00/0