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Methylation of Histidine-48 in Pancreatic Phospholipase A₂. Role of Histidine and Calcium Ion in the Catalytic Mechanism[†]

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ABSTRACT: It is known that His-48 is part of the active center in pancreatic phospholipase. To further elucidate the role of histidine-48 in the active center of pancreatic phospholipase A₂, we have modified the enzyme with a number of bromo ketones and methyl benzenesulfonates. Rapid methylation occurred with methyl *p*-nitrobenzenesulfonate. Methylated phospholipase shows total loss of enzymatic activity whereas binding of substrate and the cofactor Ca²⁺ remains intact. Amino acid analysis of methylated equine phospholipase showed the loss of the single molecule of histidine and the formation of one molecule of 2-amino-3-(1-methyl-5-imidazolyl)propanoic acid (1-methylhistidine). Equine phospholipase was also modified by [¹³C]methyl *p*-nitro-

benzenesulfonate and the methylated enzyme was studied by ¹³C NMR. The results indicate that the proton on the nitrogen in position 3 of the imidazole ring is involved in a strong interaction with a buried carboxylate group, thereby hindering rotation of the imidazole ring, and that the nitrogen in position 1 is involved in catalysis. These data are in full agreement with the three-dimensional structure at 1.7-Å resolution of bovine pancreatic phospholipase. A catalytic mechanism is proposed in which a water molecule which is close to the nitrogen at position 1 of the imidazole ring of the Asp-99-His-48 couple acts as the nucleophile. A comparison is made between phospholipase A₂ and the serine esterases.

Phospholipase A₂ (EC 3.1.1.4) has been isolated from several venoms as well as from pancreatic tissue or juice (Rosenberg, 1979). From the latter source the enzyme is isolated as a zymogen which in contrast to the phospholipase does not degrade natural phospholipids in aggregated structures (de Haas et al., 1971; Pieterse et al., 1974). Synthetic short-chain lecithins, however, are hydrolyzed at concentrations below the cmc¹ not only by pancreatic or snake venom phospholipases but also by the zymogens (Pieterse et al., 1974). From these studies it was concluded that pancreatic phospholipases and their zymogens possess an active site with comparable efficiency. The conclusion was supported by inhibition experi-

ments with *p*-bromophenacyl bromide which alkylates His-48 with the same velocity in both enzyme and zymogen (Volwerk et al., 1974). The introduction of the *p*-bromophenacyl group completely abolishes enzymatic activity and binding of monomeric analogues. Moreover, the binding of the essential cofactor Ca²⁺ is severely distorted. The conclusion of Volwerk et al. (1974) that His-48 is an active center residue is supported by the finding of several investigators that also in snake venom phospholipases modification of His-48 is accompanied by loss of enzymatic activity (Fohlman et al., 1979). Although the data support the importance of His-48 which is preserved in the primary structure of all vertebrate phospholipases, they do not specify its catalytic role.

The participation of His-48 in catalysis would be more readily demonstrated by the introduction of a small modifying group neither perturbing Ca²⁺ binding nor substrate binding and by the measurement of the pK of His-48 in the Michaelis

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¹ Abbreviations used: cmc, critical micellar concentration; 1-methylhistidine (π -methylhistidine), 2-amino-3-(1-methyl-5-imidazolyl)propanoic acid; 3-methylhistidine (τ -methylhistidine), 2-amino-3-(1-methyl-4-imidazolyl)propanoic acid; thialaminine, S-[2-(*N,N,N*-trimethylamino)ethyl]cysteine; thialaminated phospholipase, phospholipase A₂ in which all cysteines have been converted into thialamines by reduction and alkylation; Hepes, 2-[4-(2-hydroxymethyl)-1-piperazine]ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

complex and its comparison with the pH-activity profile.

Chymotrypsin has been shown to be inhibited by the substrate analogue methyl *p*-nitrobenzenesulfonate which resulted in the formation of inactive chymotrypsin in which His-57 was methylated at the 3 position (Nakagawa & Bender, 1970). The modified protein was found to bind to protein inhibitors with high affinity (Fujiwara & Tsuru, 1978). Furthermore, it has been shown that the modified enzyme still binds to small substrates and proflavin and that the dramatic lowering of the enzymatic activity is due to disruption of the charge-relay triad (Henderson, 1971).

Searching for reagents to introduce a small modifying group on His-48, we have tested a number of alkyl and aryl halo ketones as well as methyl *p*-methyl- and methyl *p*-nitrobenzenesulfonate for their ability to inhibit phospholipase A₂ activity. Rapid inhibition of equine phospholipase A₂ was observed with methyl *p*-nitrobenzenesulfonate.

In the present paper we will describe the chemical, spectroscopic, and structural data pertaining to the supposed role of His-48 in catalysis. Furthermore, we propose a catalytic mechanism for phospholipase A₂ which might account for the specific role of Ca²⁺ in catalysis and the positional and stereospecificity of the enzyme.

Experimental Procedure

Enzymes. Isolation of the precursors of bovine, equine, and porcine phospholipases and their conversion into the active enzymes was done according to published procedures (Nieuwenhuizen et al., 1974; Evenberg et al., 1977a; Dutilh et al., 1975). When methylated precursor was converted to methylated phospholipase by limited trypsinolysis, the release of the N-terminal peptide was followed by the decrease of fluorescence (Abita et al., 1972). Protein concentrations were calculated either from the absorbance with $E_{280}^{1\%}$ values of 12.3 for bovine and equine phospholipase and 13.0 for the porcine enzyme or from amino acid analyses using the values of arginine or glycine as integers. Enzymatic activity was routinely determined by titration of egg yolk emulsions (Nieuwenhuizen et al., 1974) or by spectrophotometric assay of activity on monomeric 1,2-dihexanoyldithioprolylphosphocholine (Volwerk et al., 1979).

Inhibitors. Methyl *p*-nitrobenzenesulfonate (Aldrich) was recrystallized from methanol, whereas methyl *p*-methylbenzenesulfonate (methyl *p*-toluenesulfonate, Aldrich) was used without further purification. Alkyl and aryl bromo ketones were prepared from the corresponding acyl chlorides by reaction with diazomethane, followed by reaction with a concentrated HBr solution using a procedure essentially the same as the one described by Visser et al. (1971) for the synthesis of halo ketone peptide derivatives. Products were purified by silicic acid column chromatography using toluene-acetone mixtures as eluants. *N*-(Bromoacetyl)benzylamine was prepared by slow addition of a solution of 0.5 g of benzylamine and 0.5 mL of pyridine in 5 mL of dry toluene to a solution of 1 g of bromoacetyl bromide in 10 mL of dry toluene at 0 °C. The precipitated product was collected, washed with toluene, and recrystallized from methanol-water: mp 106–109 °C (uncor).

¹⁴C- or ¹³C-labeled methyl *p*-nitrobenzenesulfonates were prepared from *p*-nitrobenzenesulfonyl chloride (Aldrich; 3 times recrystallized from hexane) and labeled methanol obtained from Amersham, England, and Merck Sharp & Dohme, Canada, respectively, according to the method of Morgan & Cretcher (1948). The specific radioactivity of the products was determined directly by using an ϵ_{253} of $13.1 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (Nakagawa & Bender, 1970) and indirectly by meth-

ylation of cysteine and determination of methylcysteine on the amino acid analyzer. Both methods gave identical values within 5% (310 dpm/nmol).

Measurement of Radioactivity. Radioactivity was determined with a Searle Isocap 300 liquid scintillation system (Nuclear Chicago Division) in a water-miscible scintillation solution (Packard Instagel). Radioactive peptides were located on paper with a spark chamber (Birchoven Instruments, Hitchin, Herts, England) as described by Smith et al. (1973).

Modification of Proteins. Unless otherwise specified, modification of proteins was done in 50 mM cacodylate buffer, pH 6.0, at 30 °C (halo ketones) or 40 °C (sulfonate) at a protein concentration of 1–2 mg/mL [$(7\text{--}14) \times 10^{-5} \text{ M}$]. The inhibitors were added from stock solutions in acetonitrile; the final concentration of acetonitrile was ~2 volume %. Modifications were stopped by acidification with acetic acid (pH 4.5), followed by cooling to 4 °C. Acetonitrile was added to dissolve any precipitate, and the solution was passed through a column with Sephadex G-25 coarse (Pharmacia, Uppsala) with 1% acetic acid as the eluant. The protein peak was collected and lyophilized. Further purification was achieved by ion-exchange chromatography on CM- and DEAE-cellulose (Whatman, England); for experimental details see Results.

Spectroscopic Measurements. Fluorescence spectra were recorded at 25 °C with a Perkin-Elmer MPF3 spectrofluorometer using 1-cm cells and a thermostated cell holder. The setting of excitation and emission slit width was 7 nm, and excitation was performed at 295 nm. In the fluorometric titration experiments, a 10–15 mL protein solution ($7 \times 10^{-6} \text{ M}$) in 1 mM Tris-acetate buffer containing 100 mM NaCl was titrated with 1 M NaOH. The pH was continuously measured with a combined electrode. After adjustment of the pH, 3-mL samples were taken and the spectrum was recorded. The fluorescence quantum yield was measured relative to a second protein sample at pH 4.0.

Ultraviolet difference spectra were obtained at 25 °C with an Aminco DW-2a spectrophotometer as previously described (van Dam-Mieras et al., 1975).

All ¹³C NMR spectra were obtained with a Bruker HX-360 spectrometer operating at 90.5 MHz equipped with a variable temperature unit (accuracy ± 1 °C) (SON Facility, University of Groningen, The Netherlands). By use of quadrature detection, 1000–2000 transients were accumulated with a repetition time of 0.82 s and a spectral width of 10,000 Hz. An exponential multiplication of the free induction decay corresponding to a line broadening of 6 Hz was applied to improve the signal to noise ratio. Chemical shifts were measured at 25 °C from benzene (capillary). Routinely, 1.5 mL of 1.0–1.5 mM protein solutions in 50 mM Hepes or Tris buffer containing 10% D₂O were analyzed by using 10-mm diameter tubes equipped with a plug to prevent vortexing. The pH was measured with a Radiometer pH meter (PHM 62) equipped with a Radiometer electrode (GK 2321 C) before and after each measurement. The pH was adjusted with 2 M NaOH or HCl in 10% D₂O. The curves in the plots of ¹³C chemical shifts vs. pH were calculated by using an interactive least-squares fitting procedure based on the program of Fletcher & Powell (1963).

Amino Acid Analyses. For amino acid analysis samples were hydrolyzed for 24 h at 110 °C in carefully evacuated ampules. The values for Thr and Ser were corrected for 5 and 10% loss, respectively. The amounts of *S*-methylcysteine were calculated assuming identical color yields of *S*-methylcysteine and of Ala. Histidine, *N*¹-methylhistidine and *N*³-methylhistidine were determined by using the long column of the

Table I: Second-Order Rate Constants (k_2) for the Inactivation of Porcine Pancreatic Phospholipase A₂ by Various Halo Ketones^a

no.	compd	k_2 (M ⁻¹ min ⁻¹)
I	<i>p</i> -bromophenacyl bromide	125
II	phenacyl bromide	79
III	1-bromo-3-phenylpropan-2-one	slow ^b
IV	1-bromo-4-phenylbutan-2-one	31
V	1-bromooctan-2-one	75
VI	<i>N</i> -(bromoacetyl)benzylamine	slow ^b
VII	1-bromopropan-2-one	0 ^c
VIII	1-iodoacetamide	0 ^c

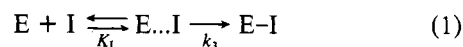
^a Conditions: 0.1 M sodium cacodylate-HCl buffer, pH 6.0, containing 0.1 M NaCl; phospholipase A₂ concentrations 7×10^{-6} M; temperature, 30 °C. The reaction was initiated by adding 10 μ L of a solution of the inhibitor in acetone to 1 mL of protein solution to give a final concentration of 10^{-4} M. The standard error in the k_2 values is about 10%. ^b Inactivation rates too slow to allow an accurate estimation of k_2 . ^c At an inhibitor concentration of 10^{-2} M the residual activity measured after 18-h incubation was more than 90%.

Technicon TSM amino acid analyzer eluted with standard buffer of pH 5.25. Under these conditions histidine and *N*³-methylhistidine overlap. Calculations were based on identical color yields for these amino acids. *N*¹-Methylhistidine and *N*³-methylhistidine standards were obtained from Sigma Chemical Co. (St. Louis, MO).

Isolation of Radioactive Peptides. Before enzymatic digestion the modified protein was reduced and the cysteines were converted into thialaminines (Evenberg et al., 1977b). The reduced and alkylated proteins were digested with trypsin (treated with tosylamidophenylethyl chloromethyl ketone) or chymotrypsin from Serva Fine Chemicals (Germany), and the resulting peptides were purified as described before (Evenberg et al., 1977b).

Results

Modification with Halo Ketones. (1) Inactivation Rates. Since *p*-bromophenacyl bromide reacts in porcine pancreas only with His-48 and not with the other histidine side chains in this enzyme (Volwerk et al., 1974), the specific reaction between halo ketones and His-48 of phospholipase A₂ can only be understood if we assume the existence of an intermediate noncovalent enzyme-inhibitor complex (eq 1).



If formation of the noncovalent complex is rapid relative to its conversion, the inactivation of the enzyme obeys the rate equation (Baker, 1976)

$$v = -\frac{d(E)}{dt} = \frac{d(EI)}{dt} = \frac{k_3(E)(I)}{K_1 + (I)} \quad (2)$$

In the case of apolar halo ketones having a low solubility and a low affinity to the enzyme [$K_1 \gg (I)$], eq 2 reduces to

$$v = \frac{k_3}{K_1}(E)(I) = k_2(E)(I) \quad (3)$$

where $k_2 = k_3/K_1$ is the second-order rate constant. This equation states that the rate of inhibition is proportional to the concentration of I. This was indeed observed with all inhibitors used in this study.

Table I summarizes the values of k_2 for the inactivation of porcine pancreatic phospholipase A₂ by various halo ketones. All compounds containing a hydrophobic moiety (compounds I-IV) inactivate the enzyme with a concomitant loss of histidine as was originally found for *p*-bromophenacyl bromide,

Table II: Amino Acid Analyses^a of Equine Pancreatic Phospholipase A₂ Inhibited with *N*-(Bromoacetyl)benzylamide at pH 7.0 and 40 °C (A) or Inhibited with Methyl *p*-Nitrobenzenesulfonate at pH 6.0 and 40 °C (B)

	% inactivation	residues per mol		
		His	<i>N</i> -1 derivative	<i>N</i> -3 derivative
(A) alkylated phospholipase A ₂	90	0.35	0.6-0.7	0
(B) methyl-phospholipase A ₂ (after purification)	99	0.05 ^b	0.96	<i>b</i>

^a The recovery of histidine and *N*-carboxymethyl derivatives is expressed as residues per mole of protein and was calculated according to Crestfield et al. (1963) using valine and arginine as internal standards. *N*¹- and *N*³-methylhistidine and histidine were calculated by assuming identical color yields for these amino acids and using the value of arginine as the internal standard. ^b His and 3-methylhistidine do not separate under these conditions.

whereas compounds VII and VIII lacking a hydrophobic part fail to inactivate the enzyme. It must be emphasized in this respect that k_2 is a composite of two other constants (viz., k_3 and K_1) which cannot be independently measured because of the poor solubility of the halo ketones. A comparison of the values of k_2 listed in Table I is only meaningful if one assumes that the hydrophobic compounds I-VI have a comparable albeit poor affinity for the enzyme.

It is interesting to note that an aromatic ring is not a structural requirement for the inhibitor, 1-bromooctan-2-one being about as equally reactive as phenacyl bromide. Introduction of an extra CH₂ group in between the bromo ketone group and the phenyl ring (compound III) or an amide bond (compound VI) leads to a considerable decrease in the reactivity of the inhibitor, whereas reactivity is largely restored by inserting two CH₂ groups (compound IV). A possible explanation for this effect might involve a difference in the fraction present in the enol rather than in the reactive keto form, although steric effects cannot be excluded.

(2) *Site of Alkylation.* Although *N*-(bromoacetyl)benzylamine (compound VI) inactivates phospholipase A₂ only slowly (Table I), complete inactivation could be achieved by allowing the reaction to proceed for 24 h at pH 7.0 and 40 °C with 100-fold excess of reagent. Acid hydrolysis in 6 N HCl generated carboxymethyl derivatives which could be determined quantitatively by amino acid analysis.

Table II, row A, shows the results of the amino acid analysis of equine phospholipase A₂ collected after 90% inhibition was reached. As shown, only the *N*¹-carboxymethyl derivative of histidine was found; no *N*³ derivative was detected. Therefore, we conclude that phospholipase A₂ is alkylated by halo ketones exclusively at the *N*¹ nitrogen. The same conclusion was drawn for methylated phospholipase (Table II, row B; see also next section).

*Reaction with Methyl *p*-Toluenesulfonate and Methyl *p*-Nitrobenzenesulfonate. (1) Rate of Inactivation.* Phospholipase A₂ is inactivated at pH 6.0 and 40 °C by using a 10-50-fold excess of methyl *p*-nitrobenzenesulfonate. The rate of inactivation of phospholipases from bovine, equine, and porcine pancreas is shown in Figure 1; the rate of inactivation obtained with methyl *p*-toluenesulfonate is at least 1 order of magnitude slower.

The inactivation did not follow pseudo-first-order kinetics due to the instability of the reagent for which a half-time of 100 min at pH 7.5 and 25 °C has been reported (Willadsen et al., 1973) and also due to nonspecific side reactions with the protein (see also Site of Methylation). For these reasons

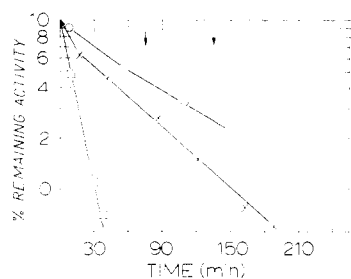


FIGURE 1: Inhibition of equine (\square), porcine (\times), and bovine (\circ) pancreatic phospholipase A_2 with methyl *p*-nitrobenzenesulfonate at 40 °C and pH 6 (for experimental details see Experimental Procedure). Protein concentration, 10^{-4} M; initial inhibitor concentrations, 7×10^{-3} M. Final concentration of acetonitrile was 2%. The arrows indicate the moments at which additional reagent was added to the solution of bovine and porcine phospholipase A_2 .

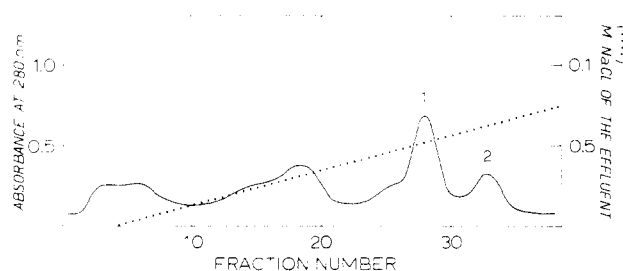


FIGURE 2: Separation of 201 mg of methylated equine phospholipase A_2 on a DEAE-cellulose column (2×58 cm). Buffer: 5 mM imidazole-acetate, pH 6.8. The protein was eluted with a linear salt gradient from 0 to 0.2 M NaCl with 80 mL of buffer in the mixing beaker. Fractions of 8 mL were collected.

no attempts were made to analyze the kinetics of the reaction or to study the protective effect of substrate analogues or the essential cofactor Ca^{2+} .

(2) *Isolation of Methylated (Pro)phospholipase A_2* . Equine phospholipase A_2 was inhibited by a 70-fold molar excess of ^{14}C reagent as described under Experimental Procedure. After reaching a residual activity of 13%, excess reagent was removed by Sephadex gel filtration (see Experimental Procedure). When the lyophilized protein was chromatographed on a DEAE-cellulose column, a rather complex picture was obtained (Figure 2). The basic proteins, which elute ahead of peak 1, were not characterized further. Peak 2 represents unmodified enzyme. Peak 1 was rechromatographed on a CM-cellulose column at pH 5.2 in order to remove traces of a contaminant which appears as a shoulder ahead of peak 1 in Figure 2. The basic fraction thus obtained (yield 35%) had 1% residual activity, moved as a single band on disc gel electrophoresis at pH 8.5, and showed an incorporation of 1.05 mol of [^{14}C]methyl per mol of protein. The amino acid composition was indistinguishable from that of unmodified phospholipase, except for the disappearance of histidine and the appearance of 1 mol of N^1 -methylhistidine per mol of protein (Table II, row B). Similar results were obtained with porcine pancreatic phospholipase. For the bovine enzyme the yields of purified enzyme were considerably lower due to a larger extent of side reactions.

Since evidence was obtained that methylation of the N-terminal Ala was a side reaction (see Site of Methylation), the modification was carried out also on the equine precursor when a ^{13}C label was introduced. The inactivation rate was comparable to that of phospholipase. An elution pattern was obtained which was slightly less complex than the one shown in Figure 2. The yield of pure [^{13}C]methylphospholipase A_2 was 45%. Limited trypsinolysis (see Experimental Procedure) converted the zymogen into [^{13}C]methylphospholipase

Table III: Binding Constants of Porcine and Equine Phospholipases with Ca^{2+} and Monomeric and Micellar Substrate Analogues^a

source of phospholipases	K (mM)					
	Ca^{2+}		$C_{10}PN$		$C_{16}PN$	
	native protein	modified protein	native protein	modified protein	native protein	modified protein
porcine	2.6	9.1	1.6	1.5	1.8	1.8
equine	1.0	2.4	20	20	5	5

^a The binding constants are determined by ultraviolet difference spectroscopy (van Dam-Mieras et al., 1975). Conditions used are as follows: buffer, 5 mM sodium acetate containing 100 mM sodium chloride, pH 5.95; protein concentration, 3.5×10^{-5} M. Substrate analogues used are monomeric *n*-decanoylphosphocholine ($C_{10}PN$) and micellar *n*-hexadecylphosphocholine ($C_{16}PN$).

which was finally purified by CM-cellulose chromatography at pH 5.2 (Evenberg et al., 1977a). Amino acid analyses of methylphospholipase as well as of methylated phospholipase demonstrated the disappearance of histidine and an appearance of 1 mol of N^1 -methylhistidine per mol of protein.

(3) *Site of Methylation*. Since equine phospholipase A_2 contains only one histidine residue, the data shown in Table II, row B, clearly demonstrate that inhibition is due to methylation of the active center histidine (His-48). Moreover, as was found for the reaction with bromo ketones, the N-1 nitrogen of the imidazole ring is methylated exclusively. When porcine phospholipase A_2 (containing three histidine residues) was methylated with ^{14}C -labeled reagent, the introduced label was located by isolating peptides of the purified, reduced, and S-alkylated protein. The major amount of radioactivity (90%) was found to be associated with a peptide analyzing correctly for the active center peptide Cys-44-Arg-53. A minor radioactive spot (10%) appeared to correspond with the peptide Glu-His-Lys-116. Similar data have been obtained after the modification of porcine phospholipase A_2 with *p*-bromophenacyl bromide (Volwerk et al., 1974).

Although the main site of modification is at the active site, there are side reactions as indicated by the amount of radioactivity (1.3–1.5 mol/mol of protein) incorporated into phospholipase at 5–15% residual activity and by the elution pattern obtained during purification of modified protein (Figure 2). The basic proteins eluting ahead of peak 1 (Figure 2) were found to have incorporation levels above 1. Since part of the label was lost upon acid hydrolysis, 6 N HCl (110 °C; 20 h), or alkaline hydrolysis (pH 10.5; room temperature; 78 h), the higher incorporation must be partly due to methylation of carboxyl groups. No attempts were made to identify the modified carboxylates.

Another site of methylation was detected in trypsin digests of unpurified [^{14}C]methylphospholipase. In addition to the histidine-containing peptides, 10–15% of the radioactivity was located in the N-terminal peptide Ala-1-Leu-Trp-Gln-Phe-Arg-6. Methylation of alanine to *N,N*-dimethylalanine was concluded from the absence of Ala from amino acid analyses and the incorporation of about two [^{14}C]methyl groups.

(4) *Substrate and Ca^{2+} Binding of the Methylated Phospholipases*. From Table III it is evident that the introduction of a methyl group in His-48 does not affect the binding of monomeric or aggregated (micellar) substrates. The binding constant for the essential cofactor Ca^{2+} is slightly affected (by a factor of 2–3) but the enzyme is still saturated with Ca^{2+} at rather low metal ion concentrations. Even though the enzyme does bind substrate and Ca^{2+} with affinities comparable to those of the native enzyme, no enzymatic activity is observed by using a micellar (egg yolk test) or monomeric

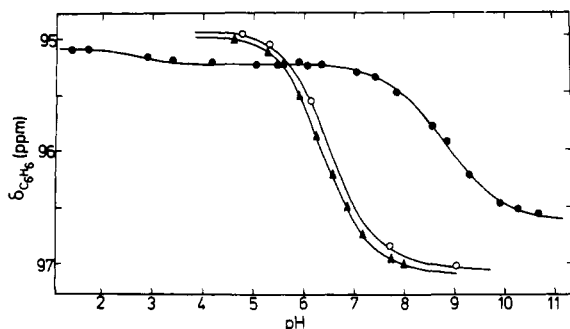


FIGURE 3: Plot of the chemical shift (ppm from C₆H₆) of the 90% enriched ¹³C nucleus in [1-¹³C]MeHis-48]phospholipase A₂ (●) and in reduced and thialaminated (○) [1-¹³C]MeHis-48]phospholipase A₂ from horse and the ¹³C natural abundance of the methyl group in N¹-methyl-L-histidine (▲) as a function of pH.

assay (Volwerk et al., 1979). Therefore, His-48 must be involved in catalyses. We will return to this point under Discussion.

(5) ¹³C NMR Measurements of [MeHis-48]phospholipase A₂. Figure 3 [curve (●)] shows a pH titration of 90% enriched [1-¹³C]MeHis-48]phospholipase A₂ from horse assayed by ¹³C NMR. An unexpectedly high pK of 8.9 is found for the deprotonation of the His-48 side chain. Because this value is close to the value reported for the α-amino group (Jansen, 1978), we could not exclude that the observed NMR signal was due to the influence of the α-amino group on the [¹³C]-methyl group of His-48. Therefore, the measurements were repeated with equine methylphospholipase, in which the α-amino group cannot play a role, and with the methylphospholipase obtained by "activation" of this modified zymogen. These proteins gave titration curves indistinguishable from the curve represented in Figure 3.

In general, substitution of the N-1 of the imidazole ring in small compounds has little effect on the pK: the free amino acid 1-methylhistidine appears to have a pK of 6.45 as shown by natural-abundance ¹³C NMR of the methyl group [Figure 3, curve (▲)]. In order to obtain additional evidence that the observed ¹³C peak originates from the methyl group on histidine, we have disrupted the disulfide bridges in the enzyme to obtain a random-coil species. In this protein the methylated His-48 has a pK of 6.65 [Figure 3, curve (○)], which is in agreement with the value of 6.45 for the free amino acid.

In the titration curves of both methylated precursor and phospholipase, a small but reproducible change in the chemical shift was observed which can be attributed to a carboxylate group with a pK of 3.2 (Figure 3).

(6) Fluorescence Measurements. Figure 4A shows the pH dependence of the fluorescence intensity of Trp-3 in equine phospholipase A₂. The increase between pH 7.5 and pH 9 reflects the deprotonation of the α-amino group. Furthermore, another group can be noticed with a pK of about 7.0. Upon addition of Ca²⁺ the pK of this group shifts to a lower value (5.5) and is therefore more clearly observed. The methylated phospholipase which still binds Ca²⁺ offers an opportunity to discriminate between His-48 and a carboxylate for the assignment of this group. Figure 4B shows the fluorescence curves for equine [MeHis-48]phospholipase A₂. Both in the absence and in the presence of 10 mM Ca²⁺ the group with a pK of 7 is not observed and therefore the group titrating with a pK of 7 is assigned to His-48. Similar observations were obtained earlier (van Dam-Mieras, 1976) with porcine phospholipase. Upon alkylation of His-48 in this enzyme with 1-bromooctan-2-one, the group with a metal ion dependent pK could no longer be observed. Because the enzyme also lost

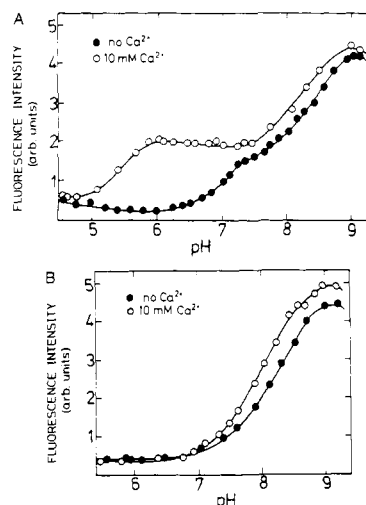


FIGURE 4: (A) Effect of pH on the fluorescence intensities (arbitrary units) of phospholipase A₂ from horse in the absence (●) and presence (○) of 10 mM Ca²⁺. (B) Effect of pH on the fluorescence intensities (arbitrary units) of [1-MeHis-48]phospholipase A₂ from horse in the absence (●) and presence (○) of 10 mM Ca²⁺.

its ability to bind Ca²⁺, the assignment of this group to His-48 was only tentative.

Discussion

The introduction of a methyl group on the N-1 position of His-48 abolishes the enzymatic activity of phospholipase A₂ but has no effects on the binding to lipid-water interfaces. Furthermore, it has no appreciable effect on the binding of monomeric substrate and Ca²⁺ to the active site. Therefore, His-48 must be involved directly in catalysis.

It has been reported (Henderson, 1971) that [MeHis-57]-chymotrypsin still has a very low activity, and solid evidence has been presented that this is an intrinsic property of the modified protein. Our purified preparations of methylphospholipase A₂ show a low (about 1%) residual activity which is probably not due to methylphospholipase but is due to the presence of a contaminant with an unmodified active site. In fact, incubation of this protein with *p*-bromophenacyl bromide abolished the residual activity.

Bromo ketones and methyl *p*-nitrobenzenesulfonate modify His-48 of phospholipase A₂ exclusively at the N-1 position (Table II). The free amino acid is alkylated more rapidly at the N³ position whereas in polyhistidine both nitrogen atoms are about equally reactive (Crestfield et al., 1963). The preferred reaction at the N-1 position indicates a strict orientation of His-48 in the enzyme with the N-1 nitrogen exposed to the solution or to the inhibitor (or substrate) when they are bound. The fixation of the imidazole side chain is effected by a strong interaction with a buried carboxylate as evidenced by the refined X-ray structure of bovine pancreatic phospholipase A₂ (B. W. Dijkstra et al., unpublished experiments) where His-48 and Asp-99 are observed at hydrogen-bonding distance (Figure 5). With this picture the group with a high pK observed in ¹³C NMR titration studies (Figure 3) can be assigned to the 1-methylhistidine side chain and the group with pK = 3 must be assigned to the carboxylate group of Asp-99.

Inspection of the X-ray structure of bovine pancreatic phospholipase A₂ also shows that two aspartic acid residues are present near His-48 while Tyr-52 is at 4 Å from His-48. Its vicinity may explain the large ring-current shift of the C₂ proton of His-48 in phospholipase A₂ observed in ¹H NMR experiments. This C₂ proton is not observed under normal

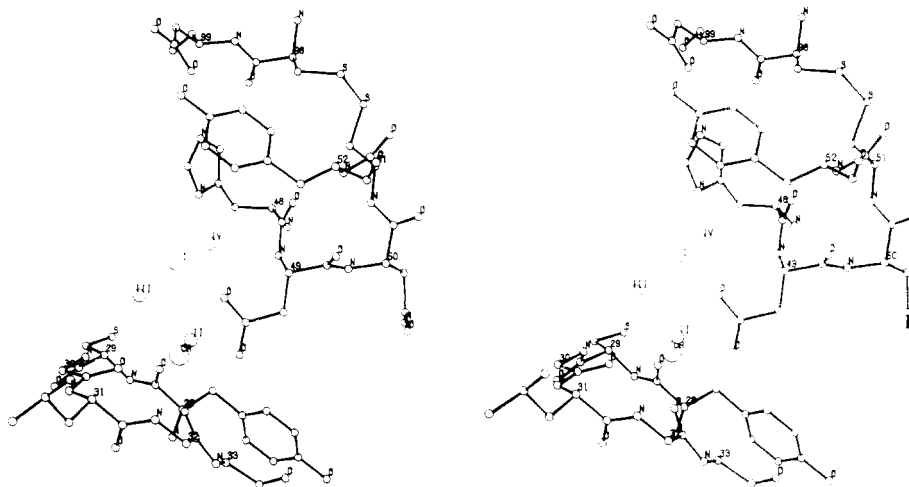


FIGURE 5: Stereoview of the active center of bovine pancreatic phospholipase A_2 . The model is derived from an electron density map at 1.7-Å resolution. The Ca^{2+} is bound to the side chain of Asp-49, to the carbonyl oxygen atoms of Tyr-28, Gly-30, and Gly-32, and to two or three water molecules labeled II and III, respectively. Water molecule II is presumably replaced by the phosphate group of the substrate in the enzyme-substrate complex. Water molecule I is supposed to perform the nucleophilic attack on the carbonyl carbon of the ester bond.

assay conditions (pH >4.6; temperature <95 °C) in the low field region of the 1H NMR spectrum of any pancreatic phospholipase A_2 (Jansen, 1978). Instead, the signal from the C_2 proton has recently been discovered under the aromatic envelope of the 1H NMR spectrum (Aguiar et al., 1979). The side chain of one of the two aspartic acid residues, Asp-49, is located at 5 Å from the imidazole ring of His-48 whereas the carboxyl group of Asp-99 is at hydrogen-bonding distance from the N-3 atom of His-48 (Figure 5).

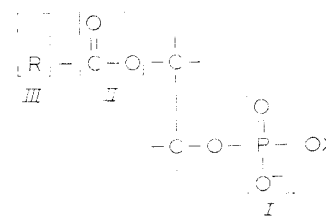
Phospholipase A_2 absolutely requires Ca^{2+} as a cofactor. The activity disappears when Ca^{2+} is replaced by Sr^{2+} or Ba^{2+} ions, even though a ternary enzyme-metal-substrate complex is formed with the latter two ions. The binding site of Ca^{2+} must be located very close to His-48 and a tyrosine residue as judged from spectral perturbations at 242 and 288 nm observed upon binding of Ca^{2+} (Pieterse et al., 1974). Binding of calcium ions to equine phospholipase A_2 shifts the pK of His-48 from about 7 to values around 5.5 (Figure 4A), indicating a charge effect at short range. Probably the slightly lower affinity of the methylphospholipase A_2 for Ca^{2+} ions at pH 6 (Table III) could be ascribed to the positive charge present at the imidazole ring of His-48 besides a possible effect on the Ca^{2+} coordination. Also, recent 1H NMR on native phospholipases showed that upon addition of Ca^{2+} ions the pK of His-48 shifts from values around 7 to values around 5.5 (Aguiar et al., 1979). From kinetic experiments (Volwerk, et al., 1979) it is concluded that catalysis depends on the unprotonated form of a group with a pK of 5.5. It seems reasonable to assign this group to the imidazole side chain of His-48.

In the preliminary interpretation of the 2.4-Å electron density map (Dijkstra et al., 1978), Ca^{2+} was assigned to a position in an electron density peak near the side chain of Asp-99. Two recent experiments, however, showed that this tentative conclusion is not correct. Fler and co-workers concluded from differential labeling experiments that specific blocking of Asp-49 abolishes Ca^{2+} binding and enzymatic activity (E. A. M. Fler and G. H. de Haas, unpublished experiments). Crystallographic refinement of the model to 1.7-Å resolution confirms that Ca^{2+} is close to one of the oxygens of the carboxyl group of Asp-49. The Ca^{2+} ion probably has six ligands: besides Asp-49, three carboxyl oxygens of the main chain (i.e., those of Tyr-28, Gly-30, and Gly-32) and water molecule II (Figure 5). These five ligands

are at a distance between 2.1 and 2.4 Å from calcium. The position of the sixth ligand is less clear, and both the second carboxylate oxygen of Asp-49 and water molecule III are to be considered (distances to the calcium ion are 2.8 to 2.9 Å).

In the absence of X-ray crystallographic data of an enzyme- Ca^{2+} -substrate (analogue) complex, one can only speculate about the spatial arrangements of such a complex. With regard to the binding of substrates or inhibitors, it has to be remarked that this binding is mainly hydrophobic in character since in compounds with one acyl chain the binding increases about threefold for every additional methylene group for chain lengths running from 6 to 12 carbon atoms (Volwerk et al., 1974). The same conclusion was drawn from kinetic experiments with pancreatic as well as snake venom phospholipase and true (monomeric) solutions of substrates carrying two acyl chains (Wells, 1972, 1974; Zhelkovski et al., 1978; Volwerk et al., 1979). This effect may also explain why inhibition with halo ketones can only be successful if the halo ketone has an apolar part (Table I; Roberts et al., 1977) which is important for binding of the inhibitor molecule to a hydrophobic area close to His-48 with the relatively polar bromo ketone function pointing to His-48. For substrate molecules this hydrophobic region may accommodate one (or both) fatty acyl chain(s).

Considering a possible mechanism for the hydrolysis of phospholipids, the minimal substrate requirements of phospholipase A_2 (van Deenen & de Haas, 1963) have to be kept in mind.



Stereospecific hydrolysis is only possible if the substrate contributes the following: I, a negative charge [phosphate or sulfate ionization (Bonsen et al., 1972)]; II, an ester bond vicinal to the site of phosphorylation; III, at least one acyl chain for hydrophobic interaction with the protein. Although Ca^{2+} ions only slightly improve substrate binding, the role of Ca^{2+} might be to bind the negative charge of the phosphate. This should fix the position of the susceptible ester bond relative

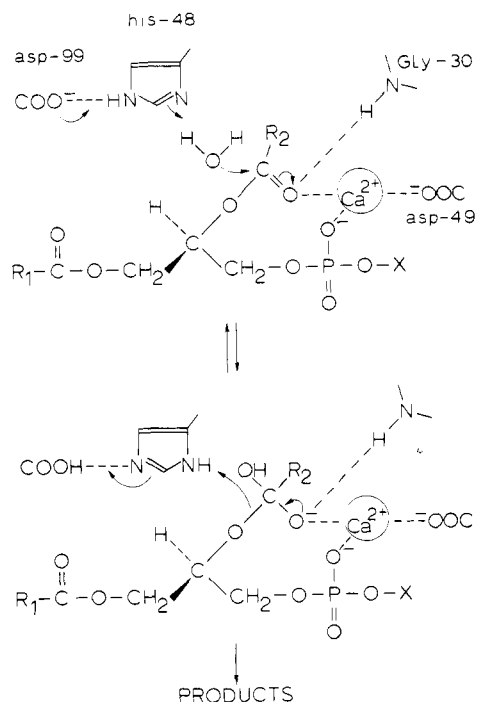


FIGURE 6: Schematic representation of a proposed catalytic mechanism of phospholipase A₂.

to the nucleophile (water molecule I in Figure 5). This function can be fulfilled by Ba²⁺ ions as well, but only with Ca²⁺ ions does hydrolysis occur. Though it is possible that the ester carbonyl carbon is shifted away from water molecule I over a distance of some tenths of an angstrom when Ca²⁺ (radius 0.99 Å) is replaced by Ba²⁺ (radius 1.34 Å) which might result in a decrease in k_{cat} , a second explanation could be that Ca²⁺ ions have a more specific role in addition to the binding of phosphate. Since Ca²⁺ is a stronger Lewis acid than Ba²⁺, it is tempting to speculate that only Ca²⁺ can effectively enhance a polarization of the ester carbonyl oxygen in the bond-making process as depicted in Figure 6.

For a discussion of the catalytic mechanism, we start with the Asp-His couple which suggests a comparison with the proteolytic serine enzymes. This comparison is not unrealistic because both phospholipase and the proteolytic serine enzymes can hydrolyze carboxylic acid esters. In the three-dimensional structure of bovine phospholipase A₂, a serine is not present near His-48, but at about 3 Å from the N-1 nitrogen of His-48 two water molecules are found in the electron density map (water molecules I and IV in Figure 5). One of these water molecules (I) lies in the plane of the imidazole ring, and this water molecule could very well perform the nucleophilic function in the ester hydrolysis in analogy to the active center serine in the esterases. When this water molecule attacks the substrate carbonyl carbon atom, the imidazole ring of His-48 picks up a proton from the water molecule, thereby facilitating the reaction. This proton is subsequently donated by the imidazole ring to the alkoxy oxygen, just as in the serine enzymes where the proton from the serine is transferred by His to the leaving group (Kraut, 1977; Komiyama & Bender, 1979). With a water molecule as the nucleophile, no acyl enzyme is involved, which is in agreement with negative results to find such an intermediate (Wells, 1971, 1972; Volwerk, 1979; Volwerk et al., 1979).

In the serine enzymes the carbonyl oxygen is bound in the so-called "oxyanion hole" (Kraut, 1977) where two hydrogen bonds from peptide NH groups fix the O atom. Looking for a similar configuration in phospholipase, we were able to fit

a substrate molecule in the active center with the susceptible ester bond in the required position with respect to water molecule I. This brings the carbonyl oxygen at hydrogen-bonding distance from the NH group of residue 30. Since the hydroxyl ion is a much weaker nucleophile than the serine anion, one could speculate that while in the serine esterases stabilization of the tetrahedral intermediate requires NH protons only, in phospholipids A₂ a more effective stabilization is achieved when the NH group of residue 30 is assisted by the charge of the Ca²⁺ ion. A similar role has been suggested for the Zn²⁺ ion present in carboxypeptidase (Quijcho & Lipscomb, 1971) and for the Ca²⁺ ion present in staphylococcal nuclease (Cotton et al., 1979).

Comparison of the primary structures of all vertebrate phospholipases A₂ sequenced so far (about 20 structures in all) not only shows an absolute conservation of His-48, Asp-49, and Asp-99 but also shows an absolute conservation of Tyr-52. The role of Tyr-52 is not yet clear. It could have the same—but thus far unknown—function as the second serine residue in the proteolytic serine enzymes where a serine residue is connected to the Asp residue of the Asp-His couple (Kraut, 1977).

The proposed mechanism may be valid both for the hydrolysis of monomeric substrates and for that of aggregated substrates. However, it does not explain the fact that the hydrolysis of the individual substrate molecules present in a lipid-water interface proceeds some 10⁴ times faster than hydrolysis of substrate molecularly dispersed in water (Verger & de Haas, 1976). This tremendous increase in speed has been explained by differences at the substrate level or by assuming conformational changes in the enzyme induced by the supersubstrate (aggregated phospholipids), this giving rise to increased k_{cat} values (Verger & de Haas, 1976). Once more a comparison can be made with the proteolytic serine enzymes where the rate of hydrolysis is highly dependent on the peptide chain length, the rate increase being as high as 10⁷ on going from an amino acid amide to a heptapeptide amide as a substrate (Bauer, 1978). This increase in rate is thought to be brought about by the interaction of the substrate with a number of subsites.

In phospholipase A₂ the supersubstrate (e.g., a micelle) could have a similar effect by being bound to the enzyme surface over a more extended area. An explanation of the enhancement in rate on a molecular basis is probably more difficult in studies using micelles (consisting of noncovalently packed monomers) rather than peptides (consisting of covalently packed monomers), and even in the latter case the molecular details of how these interactions alter the enzymatic properties are still largely unknown. A better knowledge of the precise molecular arrangement of the enzyme-micelle complex and the X-ray structures of bovine phospholipase and that of enzyme-substrate (analogue) complexes might shed more light on this problem.

Acknowledgments

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