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Phospholipase Activity of Retina and Pigment Epithelium†

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ABSTRACT: The retina proper and pigment epithelium of the bovine eye showed significant phospholipase activity at pH 4.2–4.8 and relatively low activity above pH 8.1. Phospholipases A_1 and A_2 were demonstrated in lysosomal fractions from both structures at the lower pH range while microsomes contained the A_1 form and mitochondria the A_2 form at the alkaline pH. Substrate preference and ionic requirements varied widely within the subcellular fractions. Vitamin A aldehyde and vitamin A alcohol were without effect on phospholipase activity with added substrate but the two compounds differed in their effect on release of fatty acid from endogenous substrates. No significant, consistent phospholipase activity was detected in light-adapted rod outer segments.

Phospholipase activity of two positionally specific types has been reported present in a number of mammalian tissues (Waite, 1973; Franson *et al.*, 1971; Cooper and Webster, 1970; Blaschko *et al.*, 1967; de Haas *et al.*, 1971; Ottolenghi, 1964; Gatt, 1968). Enzymes hydrolyzing β-glycerophosphate or aryl sulfate and demonstrating phospholipase-like activity have been studied in preparations of retina and pigment epithelium (Eichner, 1958; Lessell and Kuwabara, 1964; Abraham *et al.*, 1969; Ishikawa and Yamada, 1970; Burden *et al.*, 1971; Marshall and Ansell, 1971; Magalhaes and Coimbra, 1972); however, the phospholipases of these tissues have not been differentiated nor characterized.

This paper reports on studies of phospholipase A_1 and A_2 activities of bovine retinal and pigment epithelial homogenates and subcellular organelles. A preliminary summary of this work has been previously presented (Swartz and Mitchell, 1973).

Materials and Methods

Bovine eyes, obtained from a local supplier, were placed in appropriate buffers at 0–4° upon removal and retina, pigment epithelium, and rod outer segments were taken immediately from nonfrozen, iced material. Rod outer segments were gently shaken off and treated according to Frank *et al.* (1973); lysosomes were isolated by the method of Sawant *et al.* (1964), and mitochondrial membrane fractions and smooth and rough endoplasmic reticulum were harvested following the sonication method of Sottocasa *et al.* (1967). Particles were sonicated for 1–4 min at 1.0–3.0 A using a 20-kHz Branson sonifier, Model 140, with an ice–salt bath to prevent heating of the particulate preparation. Nonsonicated particles were isolated using 0.25 M sucrose and conventional flotation methods with a Beckman Model L centrifuge and SW 50.1 rotor.

The following marker enzymes were used: monoamine oxidase for outer mitochondrial membrane (McCaman *et al.*, 1965), cytochrome oxidase for inner mitochondrial membrane (Smith, 1955), acid phosphatase for lysosomes (Gianetto and de Duve, 1955), and glucose-6-phosphatase (Swanson, 1955) and ribonuclease (de Duve *et al.*, 1955) for smooth and rough endoplasmic reticulum, respectively. Enzyme specific activities were calculated from the linear segment of plots of concentration of product formed *vs.* time.

Reaction mixtures contained 6 μmol of substrate emulsified in 0.1% Triton X-100, 0.01 M Tris-HCl or acetate buffer, whole homogenate yielding 5–7.5 mg of protein or subcellular fraction containing 0.1–1.0 mg of protein, and $CaCl_2$, EDTA, $MgCl_2$ (0–20 mM), or other additions to give a final volume of 2.0 ml. Alkaline and acid phospholipase activities were linear with time for 15–20 min with up to 60 μg of protein. Maximal hydrolysis (nonlinear activity), however, was noted at a protein concentration of 150–200 μg. Emulsification of substrates in Triton X-100 eliminated the lag period of hydrolysis occasionally noted under alkaline conditions when substrates were originally merely sonicated. Incubation was for 90 min at 37.5° in preliminary experiments to determine "total" hydrolysis and for 20 min at the same temperature in routine analyses. The hydrolyzed fatty acids did not inhibit the forward reaction during this incubation period as long as membrane material was present in the mixture. Emulsification of substrate in Triton X-100 was adopted as a routine procedure after it was found that sonication of substrates in

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TABLE I: Phospholipase Activity of Retinal Preparations.

Fraction	nmol of Fatty Acid Hydrolyzed/mg of Protein per min			
	pH 4.2–4.8	Conditions[a]	pH 8.1–8.5	Conditions
Whole homogenate	41.3	Dipalm PCh + $CaCl_2$	17.5	$CaCl_2$ and EDTA inhib
	51.7	PE + EDTA	19.7	PE ($CaCl_2$ and EDTA inhib)
Lysosomes	62.2	Dipalm PCh + EDTA	10.0	
Mitochondria	3.3	PE	87.7	PE + $CaCl_2$
Microsomes	12.2	$CaCl_2$	138.0	
	4.3	Heat + deoxycholate	22.4	Heat + deoxycholate
Retina	20.0		16.0	
Lysosomes	1138.0	$CaCl_2$	1.0	$CaCl_2$ and EDTA inhib
Mitochondria	109.2	PE	41.0	PE + $CaCl_2$
Microsomes	36.4		37.0	
	10.2	Heat + deoxycholate	13.3	Heat + deoxycholate
Pigment epithelium	18.0	$CaCl_2$ and EDTA inhib	2.05	
Lysosomes	1148.9	$CaCl_2$ and EDTA inhib	1.1	
Mitochondria	87.4	PE	400.0	PE
Microsomes	26.7		33.3	
	4.2	Heat + deoxycholate	12.2	Heat + deoxycholate

[a] Dipalm = dipalmitoyl; PCh = lecithin(s); PE = phosphatidylethanolamines; inhib = inhibition. Where no substrate preference or ionic requirement is denoted, lecithins were hydrolyzed at higher rates than PE with no specific divalent ions required. $CaCl_2$ and EDTA additions were at 2 mM unless otherwise stated in the text. The data given are specific activities determined under the optimal assays conditions found for each subcellular fraction, as described in the Conditions and in the text. All other conditions were as stated under Methods.

buffer gave wildly fluctuating results from preparation to preparation.

Each fraction and the homogenate from which it was obtained was subjected to purified egg, bovine, and synthetic lecithins and phosphatidylethanolamines. All substrates were subjected to thin-layer chromatography (tlc) for identification, purification, and reisolation as previously reported (Swartz and Mitchell, 1970). Solvents were redistilled prior to use.

Enzyme activity was based on net fatty acid hydrolysis of exogenous substrates, lysolecithin accumulation, and hydrolysis of endogenous, fatty acid labeled phospholipids.

Whole homogenate denotes a preparation containing an equal protein contribution (wet weight basis) by retina and pigment epithelium.

Fatty acid extraction and titration were according to Dole (1956) with addition of silicic acid to the heptane phase followed by centrifugation to remove phospholipids. Protein analysis was by the method of Lowry *et al.* (1951).

Endogenously labeled phospholipids were prepared by incubating whole homogenate or particulate fractions with 0.1 M Tris buffer (pH 7.36), 2.5 mM ATP, 2.5 mM $MgCl_2$, 250 μM CoA, 100–200 μg of bovine serum albumin, and 15 μM ^{14}C-labeled fatty acid in a total volume of 2.0 ml. The phospholipids were isolated on tlc plates, assayed using a Packard Model 7201 chromatogram scanner, eluted from the plates, and used as substrates.

^{14}C-Labeled fatty acids were obtained from Amersham-Searle Corp. of Arlington Heights, Ill.; nonlabeled fatty acids and bovine phospholipids were products of Supelco, Inc., Bellefonte, Pa.; ATP, ADP, CoA, bovine serum albumin, and *Crotalus adamanteus* venom were from Sigma Chemical Co., St. Louis, Mo. L-α-Dipalmitoyllecithin was obtained from Mann-Schwarz Research Laboratories, Orangeburg, N. Y., and other phospholipids were from Cyclo Chemical Co., Los Angeles, Calif. Triton X-100 and organic solvents were products of J. T. Baker Chemical Co., Phillipsburg, N. J.; β-glycerophosphate, sodium deoxycholate, and other chemicals were from Fisher Scientific Co., Pittsburgh, Pa.

Experimental values represent mean averages of not less than six experiments in duplicate. Values given for enzymatic studies have been corrected for nonenzymatic hydrolysis of substrates.

Results

Nonsonicated retinal preparations showed high phospholipase (phosphatide acylhydrolase, EC 3.1.1.4) activity (Table I) with optimal fatty acid hydrolysis between pH 4.2 and 4.8 and over 8.1. At the lower optimal pH, lysosomes from all preparations showed high activity while microsomes showed low phospholipase activity but very high lysophospholipase activity (EC 3.1.1.5). Assigning the hydrolytic activity of the microsomal fraction to lysophospholipase(s) was based on the loss of fatty acid accumulation following inhibition of lysophospholipase by heating at 70° for 4 min (Marples and Thompson, 1960) and the addition of 5 mM sodium deoxycholate (Robertson and Lands, 1964). Mitochondrial preparations, like microsomes, showed low phospholipase activity at the acid pH. The first indication that two phospholipases were active at the lower pH was the observation that in whole homogenate dipalmitoyllecithin, in the presence of calcium, was the preferred lecithin substrate while phosphatidylethanolamines with EDTA added were hydrolyzed at an even higher rate. The requirement for added $CaCl_2$ for optimal activity with dipalmitoyllecithin as substrate at pH 4.2–4.8 is not noted with other lecithins although EDTA at below 1 mM concentration inhibits hydrolysis of all lecithins by homogenate, apparently by removing endogenous calcium. Egg and bovine

lecithins were inconsistently hydrolyzed by whole homogenate at rates up to 90% of that reported for dipalmitoyllecithin. The lecithins were chromatographically pure; a fact which does not, however, rule out the possibility that some minor contaminant could account for the enzyme preference for the particular substrate. With phosphatidylethanolamines as substrates for whole homogenate enzyme, EDTA increases fatty acid hydrolysis by approximately 20%. At the alkaline pH, $CaCl_2$ and EDTA inhibited all reactions studied with whole homogenate.

The most marked effects noted with media supplements were on addition of $CaCl_2$, at the acid pH, to microsomes from whole homogenate and retinal lysosomes. The requirement for calcium for activity in the microsomes was absolute and on addition of this ion to retinal lysosomes and phospholipid there was a 5.5-fold increase in the rate of fatty acid release. Whole homogenate lysosomes displayed the most definite substrate preference of any of the fractions tested. Dipalmitoyllecithin as substrate gave hydrolytic rates as high as 80% above other substrates. In general, mitochondrial preparations, regardless of tissue source, showed a definite preference for phosphatidylethanolamine as substrate while microsomes and lysosomes from the different sources showed no consistent pattern of substrate preference over the pH range studied. Other additions or substrate preferences noted in Table I were stimulatory to the extent of 10–20% above control levels but not essential for significant phospholipase activity. Where inhibition is denoted, fatty acid accumulation was below the level observed in homogenate–Triton controls.

The dispersion of substrates in serially increasing concentrations of oleate always resulted in greater detectable but very variable, quantities of liberated fatty acids. The oleate–substrate mixtures resembled sonicated substrate preparations in this respect.

Phospholipase specific activity of whole homogenate as a function of pH is shown in Figure 1. Through the range pH 3.5 to approximately 6.5, phospholipase activities were assayed with dipalmitoyllecithin with 2–5 mM $CaCl_2$ or phosphatidylethanolamine and 2–5 mM EDTA. At pH 6.5 to 6.8 through pH 8.8, $CaCl_2$ and EDTA were omitted from the media as the ions inhibited fatty acid hydrolysis with lecithin and phosphatidylethanolamine substrates. The inhibition with lecithins resulted in a loss of activity of 90–100% and a loss of approximately 50% with phosphatidylethanolamines.

Phospholipase and marker enzyme activities were determined after sonication and subfractionation of the original lysosomes (Sawant *et al.*, 1964; fraction IV), mitochondria, and microsomes of whole homogenate. These data, summarized in Table II, showed the mitochondrial fraction to be more contaminated by lysosomes than lysosomal fraction by mitochondria. Microsomes were not shown to be contaminated with mitochondria, by the methods used, but showed lysosomal content.

In preliminary work, there appeared to be a great loss of phospholipase(s) to the supernatant fraction, obtained from whole homogenate before sonication and without Triton treatment. Subsequent fractionation without prior freezing and thawing of the homogenate had no effect on the supernatant activity. Heating the fraction (Cooper and Webster, 1970) at 70° for 4 min reduced the specific activity threefold but treatment with sodium deoxycholate did not appreciably reduce the phospholipase activity of the fraction. Calcium did not stimulate this phospholipase. Nonparticulate (supernatant) fractions obtained after sonication of mitochondria, microsomes, and lysosomes did not show the same pattern

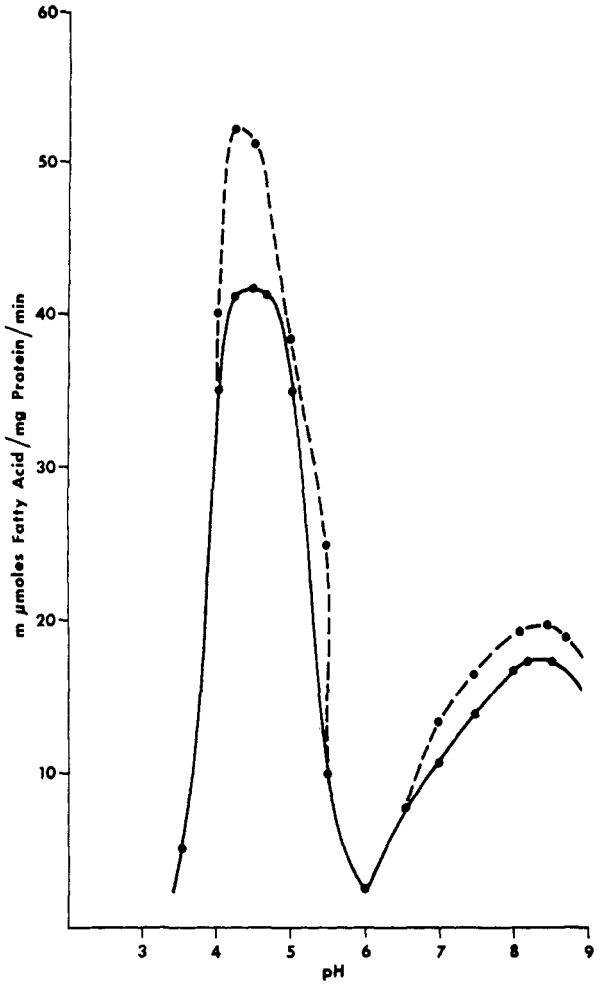

FIGURE 1: Phospholipase specific activities of whole homogenate as a function of pH. Conditions were as described in the text and under Methods: (—) dipalmitoyllecithin; (- - - -) phosphatidylethanolamine.

of response to heat, deoxycholate, or calcium as the original supernatant from homogenate. These findings suggest that the phospholipase activity of the supernatant fraction was not "lost" or leaked from other fractions. This enzyme resembles the Ca^{2+}-independent phospholipase A_2 found in rat lung supernatant (Ota *et al.*, 1972).

The sonication procedure, although allowing for the separation of soluble and particulate or membrane enzymes, did not greatly affect phospholipase activity. We attribute this finding to the solubilizing effect of the substrate emulsifier, Triton X-100, during incubation. Sonication appeared not to greatly add to the "solubilizing" effect of the Triton.

The ratio of fatty acids released to lysolecithin formed is shown in Table III. Under conditions which promoted high rates of fatty acid release (Table I), the ratios shown are near unity. Lower values were obtained under suboptimal conditions (pH) and in preparations which later proved to be nonhomogeneous by marker enzyme studies.

[^{14}C]Linoleate-labeled phosphatidylethanolamine and [^{14}C]-palmitate-labeled phosphatidylcholine were subjected to *C. adamanteus* venom and retinal particulate fractions of constant protein concentration (wet weight). The known specificity of *Crotalus* venom for the 2 position of certain phospholipids (de Haas *et al.*, 1962) and the rate of hydrolysis of substrates by the venom compared with hydrolysis by tissue preparations served as indicators of the location of the label and the

TABLE II: Phospholipase A and Marker Enzyme Activities in Fractions of Whole Homogenate.[a]

	Specific Activity					
	Phospholipase					
	pH 4–5	pH 8–8.5	Cyt *c* Oxid	MAO	G-6-P	Acid Phosphatase
	nmol/mg of protein per min[a]					
Homogenate	41.3	17.5	928.0		174.0	237.0
Lysosomes	62.2	10.0	3.0	NS	12.0	400.0
Soluble	96.2	2.0	0.9	NS		723.0
Particulate	8.5	NS	1.3	2.2	82.0	826.0
Mitochondria	3.3	87.7	1400.0	7.0	40.0	100.0
Outer membrane	3.0	92.0	401.0	50.0	110.0	100.0
Inner membrane + matrix	1.1	4.1	3200.0	5.0	20.0	6.0
Microsomes	12.2	138.0	NS	NS	210.0	279.5
Smooth ER	4.0	92.3	NS	NS	333.0	92.0
Rough ER	2.2	38.0	NS	NS	620.0	48.0
Supernatant of homogenate	89.1	67.8	NS	NS	6.2	456.0

[a] For glucose-6-phosphatase and acid phosphatase, denotes P_i liberated; for cyt *c* oxidase and MAO, substrate oxidized, and for phospholipase(s), fatty acid liberated. Fractions were not treated with Triton X-100 before assay. In the phospholipase assays, whole homogenate was supplemented with dipalmitoyllecithin + 2 mM $CaCl_2$; all fractions of lysosomes, mitochondria, and microsomes were supplied the substrates and ions found to give optimal activity in nonsonicated fractions (Table I) from whole homogenate; the substrates were emulsified in Triton X-100 as described under Methods. MAO = monoamine oxidase.

specificity of the retinal phospholipases. The specific activity (cpm/μg of lipid phosphorus) of the phosphatidylethanolamine was reduced by almost 80% by venom treatment (Table IVa), as compared to the incubated substrate without enzyme. At the lower pH, retinal lysosomes were highly active in removing the label, with pigment epithelial lysosomes showing a lower rate of hydrolytic activity at the 2 position. Mitochondria from both fractions removed a very significant quantity of the label at the higher pH but microsomes were surprisingly inactive under these experimental conditions.

Phosphatidylcholine was labeled with [^{14}C]palmitate following the preliminary observation (Table I) that lysosomes from whole homogenate preferred the unusual dipalmitoyllecithin over all other lecithins used. *Crotalus* venom phospholipases will hydrolyze saturated fatty acids at the 2 position of lecithin at a high rate but these enzymes have not been shown to have significant hydrolytic activity at the 1 position. The data presented in Table IVb indicate that the phosphatidylcholine synthesized by homogenate contained [^{14}C]palmitate in the 1 position since so little of the label was removed by the venom. Anderson and Maude (1971) found major bovine retinal phosphatidylcholine fractions containing 16.0 and 18.0 fatty acids in the 1 position and lesser fractions containing these fatty acids in the 2 position. Comparing the data of Table IVa,b, we have tentatively concluded that approximately two-thirds of the phospholipase activity of retinal lysosomes is as the A_2 enzyme and about one-third as the A_1, both active at pH 4–5. Pigment epithelial lysosomes appear to have their phospholipase activity nearly equally divided between the two enzymes at the lower pH value. Retinal and pigment epithelial microsomes showed moderate A_1 activity above pH 8.1 while mitochondria from the two tissues hydrolyzed the labeled fatty acid from phosphatidylethanolamine at a rather unexpected rate, displaying phospholipase A_2 characteristics.

We have not found consistent, significant phospholipase activity in the rod outer segments. Fatty acid hydrolysis was noted in some rod outer segment preparations but contaminating projections from the pigment epithelium could have been the enzyme source.

Initial studies in which vitamin A aldehyde or vitamin A alcohol (2×10^{-7} to 2×10^{-4} M), Triton X-100 alone, and the vitamin A compounds with added Triton or phospholipid were incubated with whole homogenate revealed no difference in the effect of the two compounds on the rate of fatty acid hydrolysis when they were added to enzyme and phospholipids. The two compounds, however, had quite different effects on the hydrolysis of endogenous phospholipids. In the absence of substrate, vitamin A aldehyde incubated with homogenate promoted the same rate of fatty acid release as homogenate–Triton "control." Vitamin A alcohol curiously depressed this endogenous activity by lowering the amount of fatty acid released by 21%, below control level.

TABLE III: Ratio of Fatty Acids Released to Lysophosphatides Formed.[a]

Fraction	pH 4–5	pH 8–9
Retina		
Lysosomes	1.08	0.83
Mitochondria	0.67	1.12
Microsomes	0.77	2.87
+ Deoxycholate		1.20
Pigment epithelium		
Lysosomes	1.04	0.87
Mitochondria	0.63	1.09
Microsomes	3.85	2.04
+ Deoxycholate	0.68	0.86

[a] Based on μmol/mg of protein using the preferred substrate and optimal requirements for each fraction as shown in Table I. Concentration of endogenous lysophosphatides subtracted to give net lyso compounds formed with added substrate.

TABLE IV: Hydrolysis of [^{14}C]Linoleate-Labeled Endogenous Phosphatidylethanolamine (a) and [^{14}C]Palmitate-Labeled Endogenous Phosphatidylchloine (b).[a]

Preparation	% Decrease in Substrate Radioactivity	
	pH 4–5	pH 8–9
Part a		
Control	0.0	0.0
C. adamanteus venom	76.3	79.7
Retina		
Lysosomes (Ca^{2+})	63.0	6.2
Mitochondria	12.0	37.0
Microsomes	3.3	12.0
Pigment epithelium		
Lysosomes (EDTA)	48.0	4.5
Mitochondria	3.6	54.0
Microsomes	7.2	6.8
Part b		
Control	0.0	0.0
C. adamanteus venom	14.2	12.0
Retina		
Lysosomes (Ca^{2+})	33.8	6.2
Mitochondria	12.0	17.0
Microsomes	3.0	27.2
Pigment epithelium		
Lysosomes (EDTA)	48.0	4.5
Mitochondria	3.6	14.0
Microsomes	12.0	26.8

[a] Experimental conditions and assay procedures are as described in Methods and Materials.

Discussion

The profile of phospholipase activity in the retina and pigment epithelium coupled with our failure to find consistent, significant fatty acid hydrolysis in rod outer segments under the present experimental conditions raises the question of the mechanism of acyl group renewal and positioning within the phospholipids of the rhodopsin-containing segments. It has been hypothesized (Burden *et al.*, 1971) that certain inherited visual cell degenerations may be initiated by lytic enzymes originating in the pigment epithelium and that such enzymes may in turn disrupt retinal lysosomes. With the pigment epithelial villi surrounding the apical portion of the rod outer segments (Yamada, 1961), the outer segments exchange nutrients with both pigment epithelium and inner segments (Young, 1969; Young and Bok, 1970) and rod outer segment disks are phagocytized by the pigment epithelium. Phospholipases of lysosomes and other particles may play a significant role in phospholipid catabolism and anabolism in the normal retina. The various lysosomal forms of the pigment epithelium may well recycle many nutrients (Hogan, 1972).

The substrate preferences and divalent ion requirements of the phospholipases studied indicate the qualitative differences of the enzymes from the various retinal fractions. More recent studies in this laboratory have shown that if a given particulate fraction, in contrast to whole homogenate, synthesizes phospholipid from labeled fatty acids and the specific particles are then presented with the endogenously formed lecithin or phosphatidylethanolamine, there is a rather high degree of positional specificity of fatty acid incorporation and hydrolysis by the various particulate fractions.

We do not place great emphasis on the preliminary finding that vitamin A alcohol, in contrast to vitamin A aldehyde, inhibits fatty acid hydrolysis in the absence of substrate. Additional experiments are needed to rule out nonspecific effects due to slight differences in the solubility characteristics of the two substances or to explore the possibility that the alcohol may have an inhibitory effect in one fraction and a stimulatory role in another.

The data presented point to significant phospholipase activity in retinal subfractions, regulated in part by divalent ions. The role of the vitamin A compounds in regulating phospholipase activity (though now obscure) and the movement and availability of divalent ions in the light- and dark-adapted eye must be elucidated before assigning any function to the retinal phospholipases other than that of fatty acid recycling and possibly, in conjunction with certain acylases, control of the concentration of lyso derivatives of the phosphatides.

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2-Chloromethyl-4-nitrophenyl (*N*-Carbobenzoxy)glycinate. A New Reagent Designed to Introduce an Environmentally Sensitive Conformational Probe near the Active Site of Papain†

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ABSTRACT: A specific quasisubstrate, 2-chloromethyl-4-nitrophenyl (*N*-carbobenzoxy)glycinate (Z-Gly-ONB-Cl), was synthesized in order to introduce the environmentally sensitive 2-hydroxy-5-nitrobenzyl (HNB) conformational probe into the covalent structure of papain near its active site, through the technique of affinity labeling. Papain, which had been obtained in fully active form by affinity chromatography, exhibited similar activities toward Z-Gly-ONB-Cl and the substrate *p*-nitrophenyl (*N*-carbobenzoxy)glycinate. Identical catalytic coefficients of 6.6 sec^{-1} were obtained for the papain-catalyzed hydrolysis of *p*-nitrophenyl (*N*-carbobenzoxy)glycinate and Z-Gly-ONB-Cl, but their Michaelis constants were 6.2 and 69.2 μM, respectively. Reaction of papain, at pH 5.0, with a 200-fold molar excess of Z-Gly-ONB-Cl resulted in the incorporation of 1 mol of HNB groups/mol of enzyme. Amino acid analysis and measurements of fluorescence emission in 6 M guanidine hydrochloride indicated modification of 1 tryptophan. Cysteine residue 25 was not alkylated during hydroxynitrobenzylation of the enzyme as evidenced by sulfhydryl group titrations. Spectral properties of the bound "reporter group" were examined in a study of the group's microenvironment. The spectral characteristics of the modified enzyme in solutions of varied pH showed that the hydroxynitrobenzyl group in activated HNB–papain was exposed to a polar medium readily accessible to water. Upon interaction of the enzyme with the competitive inhibitor, benzamidoacetonitrile, the HNB group appeared to be displaced to a slightly less polar environment; similar movement was observed upon inactivation of the HNB–enzyme by mercuric ions or by iodoacetate. Circular dichroic spectra revealed a slight dissymmetry in the environment of the "reporter group" which was somewhat increased upon enzyme inactivation. These findings are interpreted as evidence that the 2-hydroxy-5-nitrobenzyl probe is bound to a tryptophyl residue (or residues) situated in a largely aqueous environment in close spatial proximity to the cysteinyl-25 residue of the active site of papain. Changes in the HNB group's spectral properties reflect changes in the state of enzyme activation and in the binding of inhibitors to the enzyme in solution.

Apart from X-ray crystallography, the most direct evidence for the presence of an amino acid residue in the active site of an enzyme has been provided by the application of active-site-directed irreversible inhibitors. A milestone in the establishment of this methodology was the introduction of the site-specific reagent, diisopropyl phosphofluoridate, which was found to combine with the active seryl residues of certain hydrolytic enzymes such as cholinesterase and chymotrypsin (Wilson *et al.*, 1950; Schaffer *et al.*, 1954). Subsequently, reagents such as L-1-tosylamido-2-phenylethyl chloromethyl ketone and its lysyl side-chain analog were designed for the affinity labeling of other nucleophilic residues present in the active sites of various proteases (Schoellmann and Shaw, 1962; Whitaker and Perez-Villaseñor, 1968; Shaw, 1972). Prior to the establishment of a covalent bond, the enzyme and such an active-site-directed irreversible inhibitor must form an enzyme–inhibitor complex (Main, 1964). In order to facilitate such binding, the reagent, which may be either a pseudosubstrate or a quasisubstrate, is designed so as to appear structurally similar to synthetic substrates for the enzymes in question.

Since their introduction, 2-hydroxy-5-nitrobenzyl halides and their derivatives have found wide application in protein modification studies (Horton and Koshland, 1965, 1972).

† From the Department of Biochemistry, North Carolina State University, Raleigh, North Carolina 27607. *Received August 27, 1973.* This investigation has been supported in part by National Science Foundation Research Grant GB-33472, and is a contribution from the Schools of Agriculture and Life Sciences and Physical and Mathematical Sciences. Paper No. 3991 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, N. C.

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