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Reaction of Woodward's Reagent K with Pancreatic Porcine Phospholipase A_2: Modification of an Essential Carboxylate Residue

Dorit Dinur, Evan R. Kantrowitz and Joseph Hajdu*

Department of Chemistry, Boston College
Chestnut Hill, Massachusetts 02167

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Summary. Reaction between N-ethyl-5-isoxazolium-3'-sulfonate, a reagent specific for carboxylate residues, and pancreatic porcine phospholipase A_2 results in complete elimination of enzymatic activity. The modification reaction is prevented in presence of hexadecylphosphorylcholine and N-palmitoylaminoethylphosphorylcholine acting as reversible phospholipase A_2 inhibitors. The decrease of enzymatic activity correlates with the modification of a single carboxylate residue. The possible catalytic role of the carboxylate is discussed and a model for the active-site is proposed.

INTRODUCTION

Phospholipase A_2 (EC 3.1.1.4.) catalyzes the specific hydrolysis of the fatty-acid ester linkage at the 2-position of 1,2 diacyl-sn-phosphoglycerides (1). In addition to its role in metabolic phospholipid turnover the enzyme is also involved in a series of physiologically vital regulatory processes (1-5). Phospholipase A_2 has been shown to be required for platelet aggregation (2), cardiac contraction and excitation (3), prostaglandin biosynthesis (4), as well as aldosterone-dependent sodium transport (5). Despite intensive investigations of its protein structure (6) details of its catalytic mechanism of action have remained to be elucidated.

Phospholipase A_2 has been isolated and purified from a variety of reptilian as well as mammalian sources (1). Structural studies of the pancreatic enzyme including x-ray diffraction of the active bovine phospholipase (7) have revealed that an active-site imidazole moiety (His-48) as well as Ca^{++} are required for catalysis (8). Recently one of us has demonstrated that an arginine residue is also essential for catalytic activity (9), yet unlike His-48 which was effectively protected by Ca^{++} against alkylation using p-bromophenacyl

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bromide (7), the reaction between phenylglyoxal and the guanidinium group of of arginine residue was only marginally effected by the metal ion bound to the enzyme (9).

Based on the observation that no active-site serine-residue was found in pancreatic porcine phospholipase A_2, we have considered the possibility that a catalytically important carboxylate might be involved in the hydrolytic cleavage of the scissile fatty-acid ester function of the substrate. In an attempt to establish the existence of such a catalytic residue we have utilized N-ethyl-5-phenylisoxazolium-3'-sulfonate (Woodward's Reagent K, WRK) a reagent which has been successfully used for rapid and specific modification of carboxylate groups in proteins (10,11).

MATERIALS AND METHODS

Materials: Phospholipase A_2 from porcine pancreas was obtained from Boehringer Mannheim. On sodium dodecyl sulfate gel-electrophoresis the enzyme ran as a single band. For chemical modification studies the enzyme was first dialyzed against 0.01M phosphate buffer pH 4.75 (11). The protein concentration was determined by the method of Lowry et al. (12). N-ethyl-5-phenylisoxazolium-3-sulfonate (WRK) was purchased from Sigma (St. Louis) and was used as received. [^{14}C]glycine ethyl ester was obtained from New England Nuclear. Hexadecylphosphorylcholine and N-palmitoyl-2-aminoethylphosphorylcholine were prepared by literature procedures (13). The structure and purity of the compounds were ascertained by NMR, TLC and for the new phospholipid analog by elemental analysis as well (13). All other chemicals were of the highest purity available.

Assay: The formation of the free fatty-acid was monitored at pH 8.0 by continuous titration (15) using a Radiometer pH-stat. The specific activity of the enzyme in this assay was found to be 330 μmole fatty acid hydrolyzed/min/mg protein.

Modification by Woodward's Reagent K: All modification reactions were carried out in 0.01M phosphate at pH 4.75 (11) under pseudo-first order conditions using excess WRK. The pH of the solution was continuously adjusted to maintain constant conditions. The extent of inactivation of the enzyme was determined by taking aliquots at various times, and diluting the sample into the assay-mixture. Similar conditions were employed when the enzyme was incubated with N-ethyl-5-isoxazolium-3'-sulfonate in presence of hexadecylphosphorylcholine, N-palmitoylaminoethylphosphorylcholine, and Ca^{++}, respectively. The number of carboxylate residues modified was determined by incorporation of [^{14}C]ethyl glycinate which was used to trap the enol ester resulting from the reaction between WRK and the carboxylic function (10). For these experiments aliquots were withdrawn from the modification reaction-mixture and quenched by dilution into 0.3M HCl. Excess reagent was then removed by dialysis at 4°C, followed by incubation of the protein with 1.0M [^{14}C]glycine ethyl ester (1.10×10^{11} dpm/mole) at pH 4.75, 25°C for 24 hrs. Extensive dialysis against 0.01M phosphate pH 4.75 was then carried out to remove the unreacted amine and each sample was then analyzed for 1) residual activity, 2) protein content, and 3) incorporation of radioactivity.

TABLE 1

The Second-Order Rate Constants for the Reaction Between N-ethyl-5-isoxazolium-3'-sulfonate and Pancreatic Procine Phospholipase A_2[a]

[WRK][b], mM	PPC[c], mM	Ca^{++}, mM	k_{obs}, min^{-1}	k_2, $M^{-1}min^{-1}$
2.5	0	0	0.066	26.4
5.0	0	0	0.126	25.2
10.0	0	0	0.248	24.8
5.0	0	30	0.347	69.3
5.0	1.0	0	0.0578	11.6[d]
5.0	3.0	0	0.0495	9.9[e]

[a]See text for reaction conditions. [b]Woodward's Reagent K. [c]N-palmitoylaminoethylphosphorylcholine. [d]50% modification. [e]40% modification.

Reaction between phospholipase A_2 and 1-(3'-dimethylaminopropyl)-3-ethyl-carbodiimide (EDC). The enzyme was reacted with this reagent under conditions similar to those used with Woodward's Reagent K.

RESULTS AND DISCUSSION

Results. The enzymatic activity of phospholipase A_2 is completely abolished on reaction with Woodward's Reagent K. At pH 4.75 in 0.01M sodium phosphate using 5 mM N-ethyl-5-phenylisoxazolium-3'-sulfonate complete inactivation is reached in 20 min. (Figure 1). Under pseudo-first order conditions, with excess WRK, the rate of the reaction exhibits strictly linear dependence on the concentration of the modifying agent (Table 1). The second-order rate constant k_2, obtained for the reaction is 25.5 M^{-1} min^{-1}, well within the range of the corresponding rates reported for specific carboxylate-modification of staphylococcal nuclease, k_2=35$M^{-1}min^{-1}$ (16) and carboxypeptidase, A k_2 = 94.2$M^{-1}min^{-1}$ (17) using the same reagent.

Because of the well-known hydrolytic side-reaction between WRK and the solvent (10,11,16) as well as the pH dependence of the formation of the carboxylic enol-ester (16) all of our kinetic studies were conducted at pH 4.75. Since we observed no modification below pH 3.5, the reaction could readily be stopped by acidification.

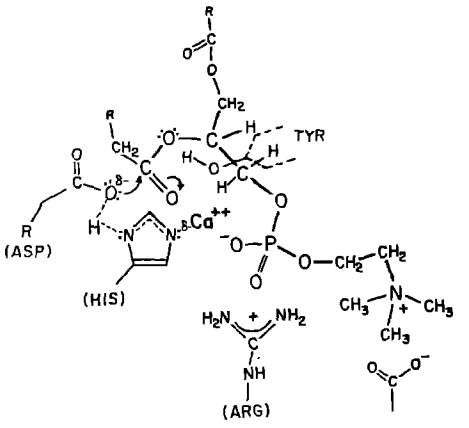

Figure 1. Reaction of N-ethyl-5-isoxazolium-3'-sulfonate (WRK) with phospholipase A_2. The reaction was monitored by the loss of enzymatic activity and the data are presented in a semilogarithmic manner. The modification reaction was carried out at 25°C in 0.01 phosphate, pH 4.75. The enzyme concentration in each reaction was 0.17 mg/ml. The residual enzymatic activity was determined by the pH-stat assay at pH 8.0. The WRK concentrations in the various runs were (A) 2.5×10^{-3} M (●) and (B) 5.0×10^{-3} M (○). Curve (C) corresponds to the reaction using 5×10^{-3} M WRK in presence of 30 mM hexadecylphosphorylcholine.

Two non-scissile phospholipid analogs were shown to be effective in protecting the enzyme against inactivation by N-ethyl-5-isoxazolium-3'-sulfonate (Figure 1, Table I). In the presence of 30 mM hexadecylphosphorylcholine as well as 15 mM N-palmitoylaminoethylphosphorylcholine no modification was observed. The aliquots withdrawn from the reaction mixture exhibited full activity upon dilution into the assay-system compared to control samples unexposed to the reagent.

Significantly, in the presence of 30 mM $CaCl_2$ the rate of inactivation of the enzyme by WRK was enhanced twofold. This is in sharp contrast to the complete protection of His-48 by Ca^{++} against alkylation using p-bromophenacylbromide (8) and the partial protection by the metal ion in the reaction with phenylglyoxal (9), targeted at the arginine residue at the active-site. The activating effect of the catalytically essential metal-cofactor in the reaction between WRK and phospholipase A_2 suggests that the modified carboxylate is likely to have a specific role in the catalytic mechanism of action of the enzyme (Figure 2).

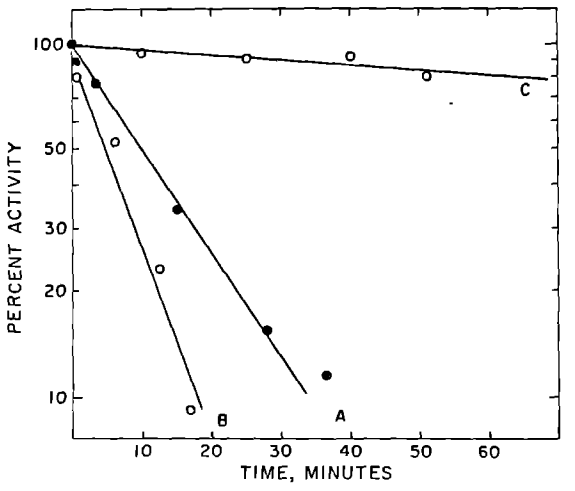

Figure 2. An active-site model for the pancreatic phospholipase A_2.

Since the product of the WRK-modified carboxyl group is a fairly reactive enol-ester (10) amino acid analysis could not be employed to determine the number of carboxylates modified. We have, therefore, utilized [^{14}C]-labeled glycine ethyl ester as a trapping agent converting the modified carboxyl residues into amide functions (10). The amount of incorporated radioactive amine per mole phospholipase A_2 inactivated by WRK was found to be one (14), indicating that the modification of a single carboxylate-residue is responsible for the loss of catalytic activity.

Additional evidence in support of the specificity of the active-site carboxylate-modification by WRK has been obtained from experiments involving reaction between phospholipase A_2 and 1-(3'-dimethylaminopropyl)-3-ethylcarbodiimide (EDC). The hydrophilic carbodiimide reacted readily with the enzyme (k_2=5.3$M^{-1}min^{-1}$) abolishing its catalytic activity, yet unlike the modification by WRK, protection was not provided against the reagent by reversible phospholipase inhibitors. It appears therefore, that inactivation of the enzyme by EDC occurs *via* covalent modification of residues other than the ones at the active site.

Discussion. The results presented in this paper clearly support the exist-

ence of a catalytically essential carboxylic side-chain in pancreatic porcine phospholipase A_2. The complete abolishment of catalytic activity on modification of a single carboxylate function and full protection against inactivation by reversible phospholipase inhibitors suggest that the modified residue is at the active site of the enzyme. In principle such a carboxylate could function, in a number of different ways (i.e., as a direct nucleophile, a general base, or a ligand to the calcium ion (16)). Of these possible alternatives, with particular emphasis on the absence of an active-site serine residue, we suggest that in phospholipase A_2 the essential carboxyl moiety is likely to function as a direct nucleophile, a role attributed to analogous residues in metalloproteases such as Glu-270 in carboxypeptidase A (19). However, in contrast to the mechanisms currently accepted for metalloproteases, in phospholipase A_2 the carboxylate group might function in conjunction with the essential imidazole side-chain, in a role similar to the strongly nucleophilic serine-residues in serine-proteases (19). Thus, calcium binding to the enzyme with concomitant proton loss to the solvent could provide the negative charge necessary for the generation of a "buried" highly nucleophilic carboxylate. The proton transfer could readily be mediated by the imidazole, serving as a bridge, accepting and releasing the proton at the same time. A similar role had been described for histidine in the well-known "charge-relay" system (19).

These proposals are incorporated into an active-site model here presented (Figure 2). Our model, intended as a working hypothesis at present, is based on structural studies including chemical modifications that have established the requirement for a histidine, an arginine, a carboxylate and for the calcium (20). The proximity between the imidazole moiety and the metal ion is clearly consistent with complete protection by Ca^{++} against chemical modification of this residue (8). Although evidence for the participation of a tyrosine residue is not as firmly established, there are indications for its involvement in the catalytic functioning of the enzyme (21).

It had first been proposed by Brockerhoff that a reactive nucleophilic group

might be involved in phospholipase A_2 catalysis (1). Notwithstanding the failure of attempts to trap possible acyl-enzyme intermediates (1), a tightly bound enzyme-substrate adduct, strongly associated with the phospholipid-aggregate (such as a micelle) may not be accessible to react with trapping agents.

Obviously further studies will be required to establish the validity of, or to modify the model here presented. Efforts in this direction, including the development of specific and potent phospholipase A_2 inhibitors are currently underway in our laboratory.

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