

Evidence for Histidine Residues on Plasma Membrane Phosphatidate Phosphohydrolase from Rat Liver

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Abstract

Objective(s)

Phosphatidate phosphohydrolase (PAP) catalyzes the dephosphorylation of phosphatidic acid to yield P_i and diacylglycerol. Two different forms of PAP in rat hepatocyte have been reported. PAP₁ is located in cytosolic and microsomal fractions and participates in the synthesis of triacylglycerols, phosphatidylcholine, and phosphatidylethanolamine, whereas the other form of phosphatidate phosphohydrolase (PAP₂) is primarily involved in lipid signaling pathways. In rat liver, PAP₂ has two isoforms; one PAP_{2a} and another PAP_{2b}. In this study, essential histidine residues were investigated in native form of rat purified PAP_{2b} with diethylpyrocarbonate.

Materials and Methods

PAP_{2b} purified from rat liver plasma membrane by solubilizing with n-octyle glucoside and several chromatography steps. Gel electrophoresis (SDS-PAGE) performed on purified enzyme in order to evaluate its purity and to measure the molecular weight of the enzyme subunit. The enzyme inactivated with diethylpyrocarbonate (DEPC) and the number of moles of histidine residues modified per mol of enzyme determined.

Results

The specific activity of purified enzyme was 7350mU/mg protein and it showed only a single band on SDS-PAGE with a MW of about 33.8 kDa. The PAP_{2b} inactivated by DEPC. The maximum 6 moles of histidine residues modified per mole of PAP_{2b}, when about 90% of enzyme activity is lost with DEPC.

Conclusion

The data showed that the incubation of PAP_{2b} by DEPC can inhibit enzyme activity. Our findings also, revealed the presence of essential histidines in the structure of PAP_{2b} which involve in its activity. This enzyme is likely to have a similar hydrolysis catalytic mechanism as its super family through a phosphohistidine intermediate.

Keywords: Diethyl pyrocarbonate, Histidine, Phosphatidate phosphohydrolase, Phosphatidic acid

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Introduction

Phosphatidate phosphohydrolase (PAP, EC 3.1.3.4) catalyzes the dephosphorylation of phosphatidic acid to yield P_i and 1, 2 diacylglycerol (1). This enzyme was first recognized as a pivotal component of metabolic pathways controlling the synthesis of glycerophospholipids and triacylglycerols (2). The diacylglycerol serves as an immediate precursor for the synthesis of major glycerolipids in animal cells (3, 4). This reaction is a regulatory step in the synthesis of triacylglycerol and phospholipids (5). Additionally, triacylglycerol (TAG) plays a key role in metabolic homeostasis, serving as the major energy storage molecule that allows organisms to survive periods of food deprivation. The regulation of TAG storage is important in human diseases because both excessive and inadequate fat storage are associated with dyslipidemia, insulin resistance, and diabetes (6-8). In rat hepatocyte, two different forms of PAP have been reported based on N-ethylmaleimide (NEM) sensitivity (9, 10). The NEM-sensitive form (PAP_1), located in cytosolic and microsomal fractions, requires Mg^{2+} for its activity and is responsible for the synthesis of phospholipids and triacylglycerols (11), whereas the other form of phosphatidate phosphohydrolase (PAP_2) is primarily involved in lipid signaling pathways by modulating the second messengers diacylglycerol and phosphatidic acid (5, 12-15). Mammalian PAP_1 is encoded by the lipin gene family which includes lipin-1, -2, and -3 (15, 16). Mutations in the mouse lipin-1 gene prevent normal adipose tissue development and lead to lipodystrophy (17). Lipin-1 was encoded by investigators (18). In human liver, there are three isoforms of PAP_2 including PAP_{2a} , PAP_{2b} and PAP_{2c} (19), whereas only two isoforms of PAP_2 , PAP_{2a} and PAP_{2b} , were identified in rat liver (5). PAP_2 does not need Mg^{2+} for its activity in comparison to PAP_1 (5, 12-14). PAP_2 has two isoforms in rat liver: one PAP_{2a} and the other PAP_{2b} . They differ in some enzymological properties (5, 14). PAP_{2a} is active against all the phosphatidic acid species, whereas PAP_{2b} is relatively inactive

against intermediate-length saturated acyl chains (5). PAP_{2a} is not purified to hemogen protein (5). The purified PAP_{2b} is inhibited by Zn^{+2} , Mn^{+2} , Ca^{+2} and Co^{+2} (4, 5). PAP_2 has been found to be related to a phosphatase super family, including bacterial acid phosphatase, diacylglycerol pyrophosphatase, yeast diacylglycerol pyrophosphatase, dihydrosphingosine-phytosphingosine phosphate phosphatase, fungal haloperoxidase, mammalian glucose 6-phosphatase, Drosophila protein Wunen, and rat Dri42 which are subsequently renamed lipid phosphate phosphohydrolase (LPPs) (20-21). There are three-domain lipid phosphate phosphatase motif in structural lipid phosphate phosphatase of *Saccharomyces cerevisiae* which are localized to the hydrophilic surface of the membrane (22-23). The sequences of this catalytic motif were investigated in *S. cerevisiae* and it was concluded that conserved arginine residue in domain 1 and the conserved histidine residues in domains 2 and 3 are essential for catalytic activity of LPP in *S. cerevisiae* (23).

The lipid phosphate phosphatase enzymes may play an important role in signal transduction by terminating signaling events of lipid phosphates, i.e., generating bioactive lipid molecules such as diacylglycerol and lysophosphatidate by LPP that initiate signal transduction events such as platelet aggregation and cell proliferation. Thus, the regulation of lipid phosphate phosphatase activities is likely to modulate the balance of the signaling molecules that are substrates and products in their reactions (23-25).

In some species, the characterizations of LPP isoforms have already been studied through gene cloning and expression (26). In this study, we have investigated the presence of histidine residues in rat purified PAP_{2b} in native form with diethylpyrocarbonate.

Materials and Methods

Materials

Phosphatidic acid (sodium salts), dithiothreitol, NEM, leupeptin, diethylpyrocarbonate, hydroxylapatite, pepstatin, Soya bean trypsin inhibitor, Affi-Gel Blue, heparin Sepharose,

low molecular mass marker, n-octyl glucoside, Sephacryl S₃₀₀ obtained from Sigma chemical Co. (USA). All other chemicals were reagent grade.

Animals

The source of rats and their maintenance was the same as the one reported by Haghighi (27).

Enzyme assay

PAP₂ activity measured in the assay buffer (250 µl) containing 50 mM Tris HCl buffer pH 7.4, 1 mM dithiothreitol (DTT), 1 mg/ml bovine serum albumin, 3.2 mM TritonX-100, 4 mM NEM, 1 mM EGTA, 1 mM EDTA, 0.35 mM phosphatidate and appropriate amount of the enzyme solution. The assay mixture incubated for 10 min at 37 °C. The reaction stopped by adding 0.5 ml trichloroacetic acid (10%). Hence, the released P_i measured (27). All assays were linear in relation to the incubation time and the protein concentrations used in them. One unit (U) of PAP₂ defined as the amount of enzyme that catalyzed the release of 1 µ mole of P_i per min under the standard assay conditions.

Purification of PAP_{2b}

PAP_{2b} was purified by buffer A (containing 25 mM Tris HCl (pH 7.4), glycerol (10%), 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 50 mM NaCl, Triton X-100 (1%, W/V), 1 mM benzamidine and 2 µg/ml of each of lupeptin, pepstatin, soya bean trypsin inhibitor) from rat liver plasma membrane as described by Fleming *et al* (5), except for the step that hydroxylapatite was done through batch wise procedure. In brief, PAP_{2b} purified in several steps from rat hepatocyte membrane including the isolation of cell membrane, releasing the enzyme from it with n-octyl glucoside detergent and, at the end of this phase PAP₂ precipitated with ammonium sulfate and subsequently loaded on Sephacryl S₃₀₀ gel filtration column. In this step, two peaks of PAP₂ including PAP_{2a} and PAP_{2b}, separated from each other, then PAP_{2b} used for batch wise with hydroxylapatite, heparine-Sepharose and Affi-Gel Blue chromatography steps. The native molecular mass of PAP_{2b} determined by gel filtration.

Kinetic studies

Kinetic constants calculated according to

surface dilution kinetic model (28). The mole percentage of phosphatidate in the mixed micelles of the Triton X-100/phosphatidate calculated using the formula (28):

$$\text{mol \%} = ([\text{PA}] (\text{bulk}) / ([\text{PA}] (\text{bulk}) + [\text{Triton X-100}])) \times 100$$

The results illustrated as a double reciprocal plot.

Inactivation of PAP_{2b} with diethylpyrocarbonate (DEPC)

The solution of diethylpyrocarbonate was freshly prepared (29). The purified enzyme (0.5 µg) separately incubated with 0, 0.2, 0.4, 0.6, 0.8 and 1 mM DEPC at 25 °C in assay buffer for 5 min prior to enzyme assay and subsequently the activity of PAP_{2b} determined and the results depicted as a graph.

Determination of the number of modified histidine residues

The modified reaction mixtures containing 80 µg/ml of purified enzyme with 0 to 1 mM DEPC in buffer A incubated at 25 °C for 5 min. By the end of incubation, the corresponding absorbance at 242 nm (30) measured with a spectrophotometer (Shimadzu Multispec 1501, Japan) and also, the enzyme activity was tested. The number of modified residues in PAP_{2b} calculated by the change in absorbance with an extinction coefficient of $3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for N-carboxyhistidyl at 242 nm (29, 30) and assuming a MW of 182 kDa for native PAP_{2b} through gel filtration by Sephacryl S₃₀₀ (2.5×43 cm). Controls incubated in the same manner with respect to the experimental samples, except the omission of DEPC. The data illustrated as a graph.

The effects of hydroxylamine on the modified PAP_{2b}

For complete inactivation of the enzyme, it (80 µg/ml) was incubated with 1 mM DEPC in buffer A for 5 min at 25 °C. To determine the hydroxylamine effect on the recovery of enzyme activity from inactivated enzyme by DEPC, in the medium 0.4 mM hydroxylamine prepared and enzyme samples removed to enzyme assay at different times (0-25 min).

Other methods

Protein concentrations determined by the method of Bradford (31), with bovine serum albumin as the standard. To evaluate purity and to measure the molecular weight of the enzyme subunit SDS poly acrylamide gel

electrophoresis (SDS-PAGE) performed with 10% slab gel as described by Tompson (32). Prior to electrophoresis, for the removal of Triton X-100 protein samples precipitated in acetone/ammonia (33). The proteins detected by silver staining (34), whereas the molecular weight of the enzyme estimated by gel filtration on a Sephacryl S₃₀₀ (2.5×43 cm) column equilibrated with buffer A. The column was calibrated with thyroglobulin (669 kDa), catalase (232 kDa), albumin (67 kDa) and chymotrypsinogenA (25 kDa).

Results

Enzyme purification

The specific activity of purified enzyme was 7350 mU/mg protein and the enzyme showed only a major single band on SDS-PAGE with a MW of about 33.8 kDa (Figure 1). The obtained K_m in the absence of DEPC was 0.45 mol %, according to surface dilution kinetic model in the presence of Triton X-100. The native enzyme exhibited an apparent molecular mass of 272 kDa on gel filtration in the presence of Triton X-100 (Figure 2). Assuming that each Triton X-100 micelle contains one enzyme molecule and that each micelle is 90 kDa in size, then the native PAP_{2b} would have a molecular mass of 182 kDa.

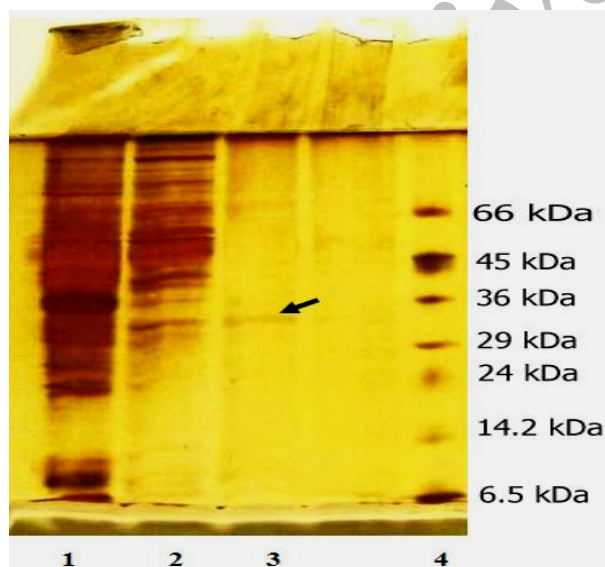


Figure 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified PAP_{2b}. Lanes 1-3 are homogenate, membrane fraction and purified enzyme, respectively. The molecular mass standard (lane 4) are, from the top, bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), lactalbumin (14.2 kDa), aprotinin (6.5 kDa).

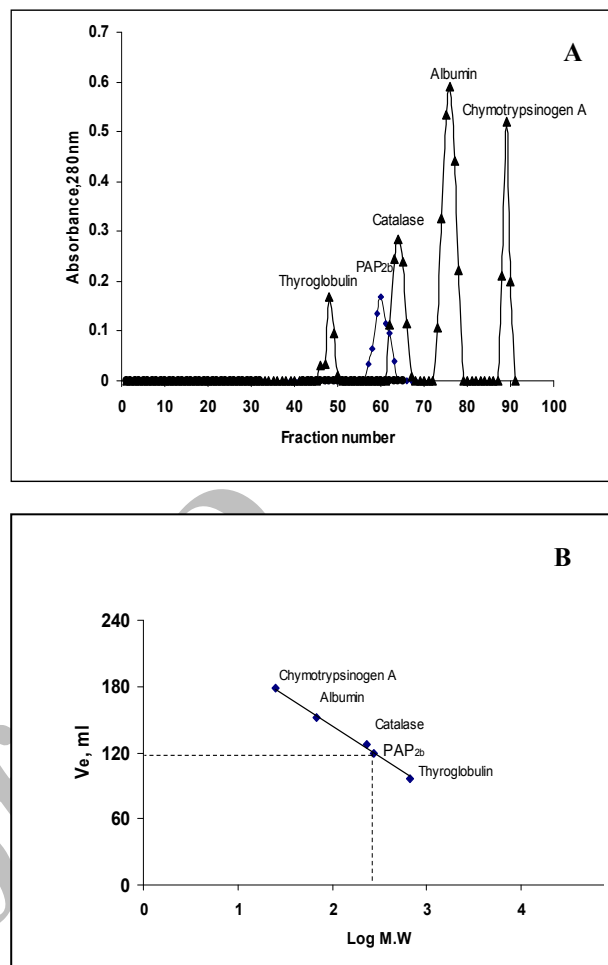


Figure 2. Gel filtration on a Sephacryl S₃₀₀ column. The column was calibrated with 10 mg/ml each of thyroglobulin (669 kDa), catalase (232 kDa), albumin (67 kDa) and chymotrypsinogen A (25 kDa). 100 µg was applied from the purified PAP_{2b}. Fractions were collected in 2 ml volumes. A; the elution profile of standard proteins and PAP_{2b}. B; the data from A were plotted against MW's. The dotted lines show the enzyme elution volume and MW of the enzyme.

Enzyme modification

The inactivation of PAP_{2b} with different concentrations of DEPC is shown in Figure 3. The pre-incubation of PAP_{2b} with DEPC (1 mM) for 5 min in the assay buffer inhibited the enzyme activity by 90 %. The increased absorbance at 242 nm due to formation of N-carboxyhistidine monitored during the course of inactivation spectrophotometrically. Figure 4 shows the absorbance of treated and untreated enzyme with DEPC at 280 nm. The absorbance of DEPC treated enzyme increased compared to that of the native enzyme.

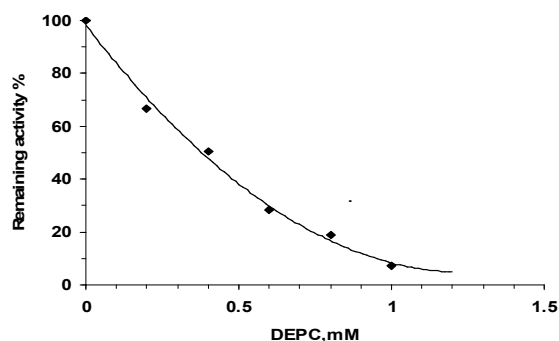


Figure 3. Inactivation of PAP_{2b} by DEPC. PAP_{2b} (0.5 µg) was incubated with different concentrations of DEPC in buffer assay for 5 min prior to enzyme assay. Each point represents the average of two independent experiments.

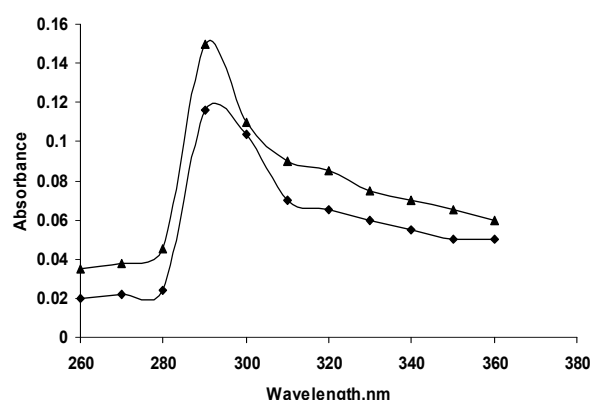


Figure 4. Absorbance change of PAP_{2b} modified with DEPC. Ultraviolet absorption spectra of native (♦) and the modified (▲) enzyme with the same concentration.

Stoichiometry of the modified PAP_{2b} with DEPC

Using extinction coefficient of $3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for N-carboxyhistidyl at 242 nm and assuming a MW of 182 kDa for native PAP_{2b} in the absence of Triton X-100 resulted in calculating six histidyl residues modified per mole of the enzyme. By extrapolating the linear part of the plot to zero activity, when 90% of the enzyme activity was lost (Figure 5). Inactivation did not proceed longer to prevent non-specific binding of DEPC.

The effect of substrate on inactivation of PAP_{2b} with DEPC

Incubation of PAP_{2b} with phosphatidate in the assay buffer prevented the inhibitory effect of DEPC on the enzyme activity in the medium (Figure 6). Whereas, in the absence of phosphatidate (substrate), DEPC led to inhibition of the enzyme activity.

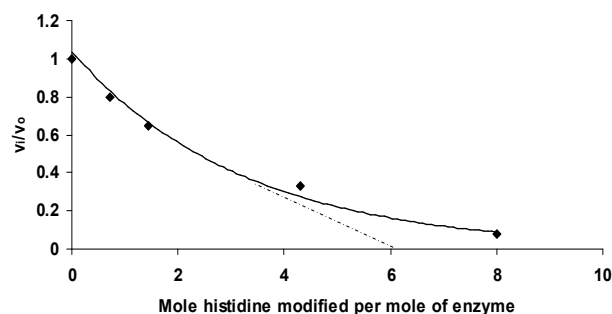


Figure 5. Correlation of the inactivation of PAP_{2b} with modification of histidine residues. The enzyme (80 µg/ml) in buffer A was incubated with different concentrations of DEPC for 5 min prior to the enzyme assay and then the number of histidine residues modified per mole of enzyme was determined as described in the methods. Values represent the average of two independent experiments.

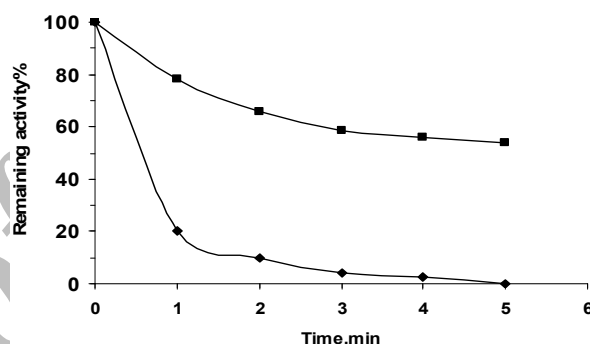


Figure 6. The protection effect of phosphatidate on the inactivation of PAP_{2b} with DEPC. PAP_{2b} activity in the presence of phosphatidate plus DEPC (each of 1mM) prior to the incubation time (■) and post incubation time with DEPC (♦).

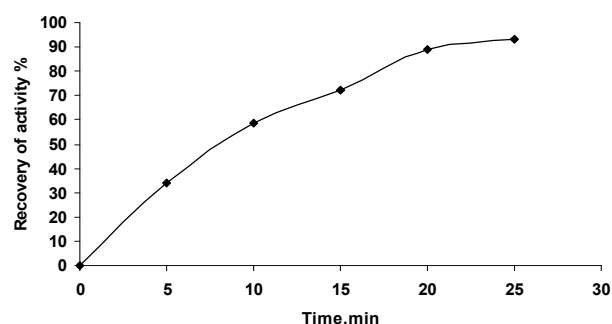


Figure 7. Hydroxylamine reactivation of DEPC-modified PAP_{2b}. Enzyme was inactivated with 1 mM DEPC followed by adding hydroxylamine to a final concentration 0.4 mM at the end of inactivation course.

The effect of hydroxylamine on the modified PAP_{2b}

Figure 7 shows the treatment of inactivated PAP_{2b} by hydroxylamine (0.4 mM final concentration in the medium) resulted in restoring the enzyme activity up to 90%. This reaction accompanied by a decrease of absorbance at 242 nm.

Discussion

The obtained K_m of PAP_{2b} is close to the value reported by other investigators (5). In this study, MW of PAP_{2b} subunit determined as 33.8 kDa, using SDS-PAGE, (Figure 1), but other investigators have reported subunit MW of 35 and 31 kDa (35, 36). Gel filtration experiment showed a MW of 272 kDa in the presence of Triton X-100 micelle (Figure 2). Assuming that each Triton X-100 micelle contains one enzyme molecule and that each micelle is 90 kDa in size (37), then the native PAP_{2b} would have a molecular mass of 182 kDa in the absence of Triton X-100 micelle agreeing with reported 175 and 186 kDa by other investigators (5, 35). The observed differences could be due to co-purification with phospholipids or the glycosylation content of PAP_{2b}. According to 33.8 kDa subunit MW of PAP_{2b}, the enzyme has probably a hexamer structure. This probable structure was reported by other investigators (35). Some enzymological characteristics of plasma membrane phosphatidate phosphohydrolase have been reported (5). However, as far as we know, the presence of histidine residues in purified rat PAP_{2b}, in native form with diethylpyrocarbonate were not studied. The present study indicated that PAP_{2b} rapidly inactivated by DEPC. The inactivation was not reversed by dialysis and was an irreversible inhibition due to covalent modification. Although DEPC primarily modifies histidine, it can also, modify lysine and tyrosine (38, 39). Carbethoxylation of histidine by DEPC resulted in an increase in absorbance at 242 nm, whereas, modified tyrosine results in a decrease in absorbance at 280 nm (40, 41). The treatment of DEPC modified protein with hydroxylamine results in removal of the ethoxyformyl group from modified histidines and tyrosines, but it does not reverse the modification of lysines (39). Figure 4 shows that PAP_{2b} modification with DEPC has resulted in an increase at 280 nm. The increased absorbance at 242 nm is correlated with the loss of activity in the modification of PAP_{2b} by DEPC, whereas, the restoration of activity, obtained by the incubation of the

inactivated enzyme with hydroxylamine (Figure 7). Hence, histidyl residues must be modified. The number of modified histidine residues can be measured spectrophotometrically (29). The stoichiometric studies of the reaction showed the modification of six moles histidine residues per mole enzyme (Figure 5). Considering a hexamer structure for the enzyme, one histidine residue could be modified per enzyme subunit. We have already reported the importance of histidine and lysine residues in PAP₁ catalysis (30, 42). The histidine residue in PAP₁ plays a general base role and involves in dephosphorylation of phosphatidate. On the other hand, results of experiments with glucose 6- phosphatase and chloroperoxidase, two members of LPPs, demonstrated that the hydrolysis of the phosphate ester occurs through a phosphohistidine intermediate (20). Our findings also, indicated the involvement of histidine in the enzyme activity of PAP_{2b}. Regarding the inhibition of PAP_{2b} with DEPC, this histidine is probably located near or in the active site of the enzyme. Thus, this enzyme is likely to have a similar hydrolysis catalytic mechanism as its super family through a phosphohistidine intermediate. In order to propose a probable mechanism for the enzyme reaction, the presence of other important amino acids such as Lys and Arg are required to investigate more in the activity of the enzyme.

Conclusion

The results of this study show that histidine residues have an essential contribution to enzyme activity in the structure of PAP_{2b}, so that the binding of DEPC to this histidine residue results in the inhibition of enzyme activity. It is likely that PAP_{2b} has a similar hydrolysis catalytic mechanism as its super family, through a phosphohistidine intermediate.

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