

MOLECULAR WEIGHT DETERMINATION AND METAL ION REQUIREMENT OF PHOSPHATIDATE PHOSPHOHYDROLASE PURIFIED FROM CYTOSOLIC FRACTION OF RAT LIVER

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Abstract

Phosphatidate phosphohydrolase (PAP) from cytosolic fraction of rat liver was purified to homogeneity having specific activity of 5.14 U/mg protein. An activity staining procedure was developed to determine molecular weight of the enzyme on polyacrylamide gel electrophoresis using Ferguson plot. Molecular Weight (M.W.) of the active PAP was 298 KDa. SDS-PAGE analysis showed a M.W. of 47 KDa for PAP subunits. Active multimer of the enzyme, therefore, was calculated to be hexamer. Gel filtration on Sephadex G-100 column showed a M.W. of 850 KDa for PAP due to the protein aggregation on the matrix. The purified enzyme was inhibited by divalent cations such as Fe^{2+} , Cu^{2+} and Ca^{2+} but requires Mg^{2+} for its activity. The activity loss of PAP inhibited by cations was restored by Mg^{2+} on polyacrylamide gel. The data suggest that the active form of cytosolic PAP is a hexamer of identical subunits and that charge density plays an important role in enzyme-substrate interaction. Magnesium ion is probably the only divalent cation capable of generating proper enzyme-metal-substrate complex necessary for the catalytic activity.

Keywords: Phosphatidate phosphohydrolase; Phosphatidate phosphatase

Introduction

Phosphatidate phosphohydrolase (EC.3.1.3.4) (PAP) catalyzes dephosphorylation of Phosphatidic acid. The cytosolic form of PAP is considered to be a key enzyme in glycerolipid biosynthesis [1]. The regulatory role of PAP [2] and some properties of the enzyme isolated from various sources [3-6] have been reported.

Structural studies have shown contradictory results for this enzyme. Carman and Ping-L reported a M.W. of 93 KDa for partially purified PAP from yeast using gel filtration technique [7]. Immunoblot analysis, however, did not prove it and suggested a 45 KDa for the yeast enzyme [8]. Membrane-associated PAP isolated from rat liver was shown to be a hexamer each monomer having a M.W. of 31 KDa using gel electrophoresis [9].

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Studies concerning metal requirement for PAP catalysis also showed contradictory conclusions [10,11]. Recently we have reported that cation-phosphatidate interaction provides suitable configuration for the substrate necessary for PAP activity [12].

In the present study, cytosolic PAP from rat liver was isolated to homogeneity.

An activity staining procedure was developed and some structural characteristics of the enzyme were investigated.

Materials and Methods

Animals

Male wistar rats (200-230 g) were received ethanol (5 g/kg) through a gastric tube 24 h before sacrifice to induce PAP synthesis [13] and deprived of food during the last 8 h before sacrifice.

Chemicals

Phosphatidic acid (sodium salt), dithiothreitol, DEAE-Cellulose, CM-Sephadex, Sephacryl S-300, hydroxyapatite, phenylmethylsulfonyl-fluoride and molecular weight markers were purchased from Sigma Co. (U.S.A.). Blue Sepharose-CL-6B was from Pharmacia (U.K.). All other chemicals were from highest grade available.

Enzyme Assay

The activity of cytosolic PAP was determined by measuring inorganic phosphate released from an aqueous dispersion of phosphatidate solution as described before [12,14]. The assay mixture was 0.1 M Tris-HCl buffer pH 7.4 containing 2.5 mM MgCl₂, 1 mM dithiothreitol, 2 mM phosphatidate, 50 μM EDTA, 100 μM EGTA, 100 μg/ml BSA and appropriate amount of the enzyme solution.

Enzyme Purification

The livers of 10 rats were separately perfused *in situ* using 0.15 M NaCl solution to remove inorganic phosphate. The livers were then removed and homogenized in 3-volume of 50 mM Tris-HCl buffer pH 7.4 containing 0.225 M sucrose and 1 mM EDTA as described before [15]. The homogenate was then centrifuged at 100,000 g for 60 min. The floating lipid was aspirated and the particle-free supernatant was decanted for the enzyme purification. The enzyme was purified according to Butterwith *et al.* [5]. The

purification procedure includes ammonium sulfate precipitation and chromatography on Sephacryl S-300, DEAE-Cellulose, CM-Sephadex, hydroxyapatite and Blue Sepharose columns.

Protein Determination

Protein was measured by the method of Lowry *et al.* [16].

Activity Staining

A procedure for activity staining of PAP on polyacrylamide gel was developed as follows. The purified enzyme (1.2 μl) as loaded on polyacrylamide gel prepared in buffer A (see below). For a 6.5% gel concentration 8 ml of 30.8% acrylamide-bisacrylamide, 0.3 ml ammonium sulfate (10%), 30 μl TEMED and 14.2 ml H₂O, were added to 15 ml of buffer A. Buffer A was 50 mM Tris-HCl pH 7.4 containing 40 μM dithiothreitol, 20 μM EDTA, 20 μM EGTA, 0.63 mg/ml phosphatidate and 13 mM ammonium molybdate. Electrophoresis was performed [17] and the gel was incubated in 50 ml of Borox-Pyrosulfite solution (52 mM Borox and 95 mM sodium pyrosulfite) for 30 min at 37°C. Hence, the gel was placed in 50 ml of hydroquinone-ascorbate solution (200 and 20 mg/ml, respectively) until the blue band corresponding to the enzyme activity appeared. Immediately after color development the gel was taken off for photography.

Molecular Weight Determination

Molecular weight of active multimer enzyme was determined by polyacrylamide gel electrophoresis in the absence of SDS using activity staining procedure described above and Ferguson plot as described by Bryan [18]. Multimeric M.W. was also determined by gel filtration on Sephadex G-100 column. The M.W. markers ranging from 31 to 1000 KDa were used for both experiments.

The Effects of Bivalent Cations

PAP activity was measured in the presence of Mg²⁺, Fe²⁺ or Ca²⁺ at a concentration range of 0-8 mM.

Results

Enzyme Purification

PAP from cytosolic fraction of rat liver was purified up to a 400-fold as described in the methods. Specific

activity of the purified enzyme was 5143 nmol p_i /min/mg corresponding to that reported before [5].

Molecular Weight Determination

Gel filtration experiments showed a M.W. of 850 KDa for PAP. This may be resulted from enzyme aggregation on Sephadex matrix (see discussion). The enzyme aggregation may generate different enzyme forms some of which having enzyme activity (Fig. 1).

SDS-PAGE showed a M.W. of 47 KDa for PAP subunits. The M.W. of the active catalytic form of PAP was determined by polyacrylamide gel electrophoresis and staining the gel for the enzyme activity. A typical electrophoregram of activity staining for 6 to 10% gel is shown in Figure 2.

Molecular weight markers of thyroglobulin, egg's albumin and myosin were also electrophoresed under the same conditions but stained by Coomassie blue (data not shown). Relative mobility of each band (Rm)

measured and log Rm values plotted against gel concentration (Ferguson plot) (Fig. 3). The slopes of the lines were then plotted against M.W. of the corresponding markers (Fig. 4) from which M.W. of the active PAP was determined to be 298 KDa. Dividing this value by subunit M.W. (47 KDa) showed the active unit of liver cytosolic PAP to be hexamer.

The Effects of Cations on PAP Activity

The purified PAP required Mg^{++} for its activity and no activity appeared in the absence of this cation. Other cations such as Cu^{2+} , Fe^{2+} and Ca^{2+} did not significantly affect the enzyme activity but at concentrations above 4 mM exhibited enzyme inhibition compared to the activity obtained in the presence of Mg^{2+} (Fig. 5).

Polyacrylamide gel electrophoresis of PAP showed that the activity lost upon staining in the presence of 5 mM Ca^{2+} , was restored by incubating the gel in the presence of 2 mM Mg^{2+} (Fig. 6).

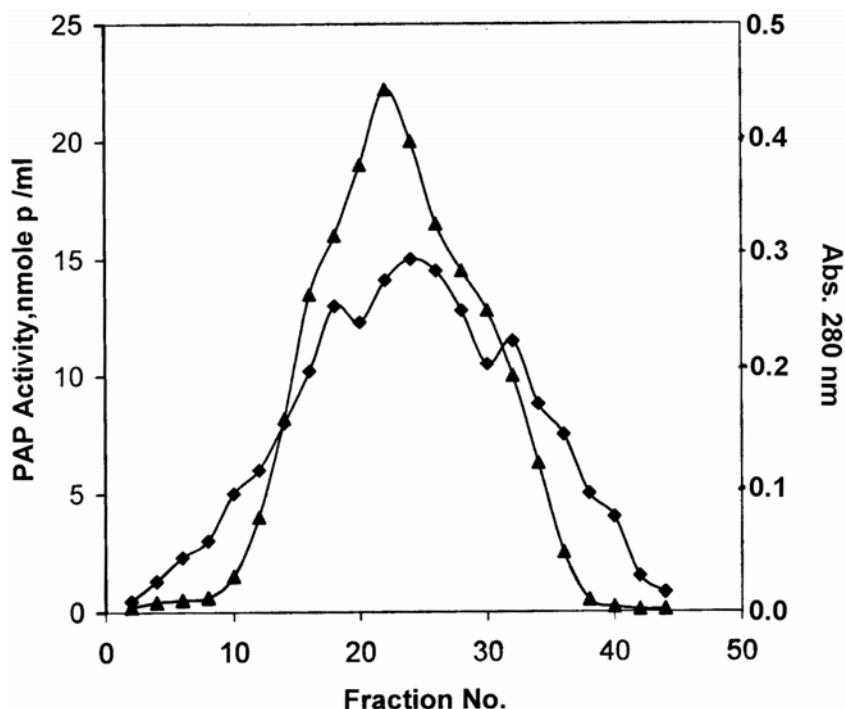


Figure 1. Gel Filtration chromatography of purified PAP on Sephadex G-100. Column (1.5×95 cm) was packed with Sephadex G-100 and equilibrated with 0.05 M Tris-HCl buffer pH 7.4 containing 1 mM EDTA, 0.2 mM dithiothreitol and 3.5% glycerol. Enzyme (70 μ g) was applied and eluted with the same buffer. Similar columns were prepared for the M.W. Markers.

▲, enzyme activity. ◆, absorbance at 280 nm.

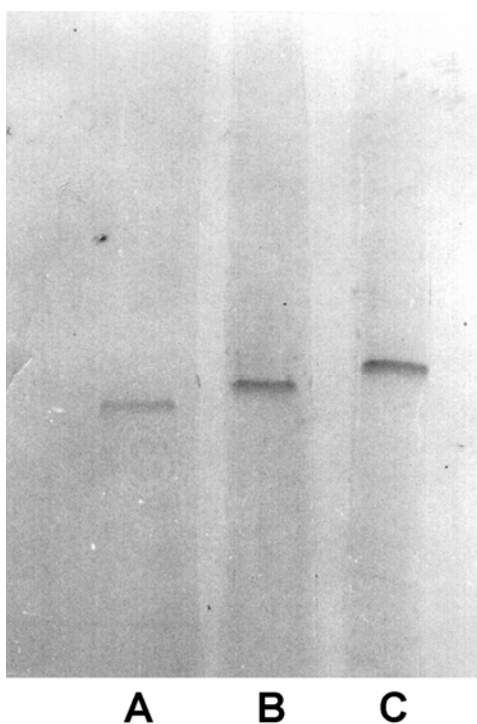


Figure 2. Electrophoresis of PAP on polyacrylamide gel. The gel at concentrations (%T) of 6% (A), 8% (B) and 10% (C) prepared and 0.24 μ g of the enzyme applied for each concentration. Electrophoresis and activity staining were performed as described in Methods.

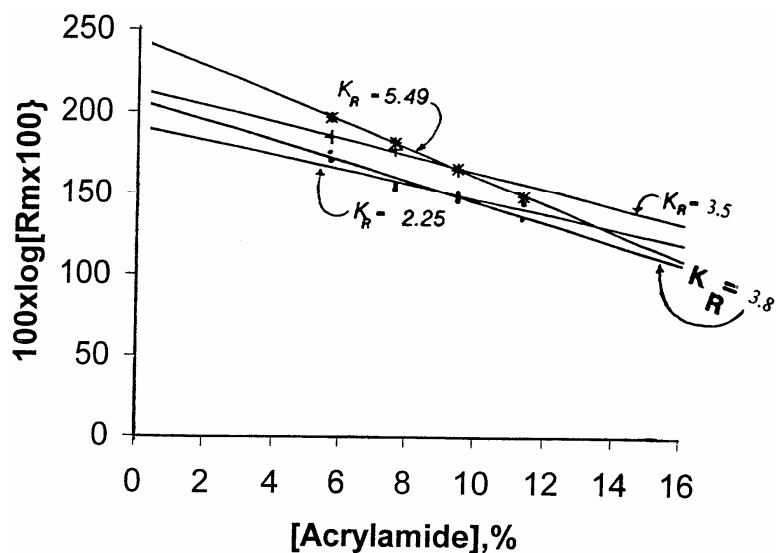


Figure 3. Ferguson plot for PAP (+) and molecular weight markers of egg albumin (■), Thyroglobulin (*) and myosin (●). For each marker 10 μ g and for PAP 2.4 μ g was applied on polyacrylamide gel (6-10%). The bands of markers were stained with Coomassie Blue and that of PAP stained for the enzyme activity. Each point is the average of two different experiments. The R_m values were plotted against the gel concentration. For detail see text.

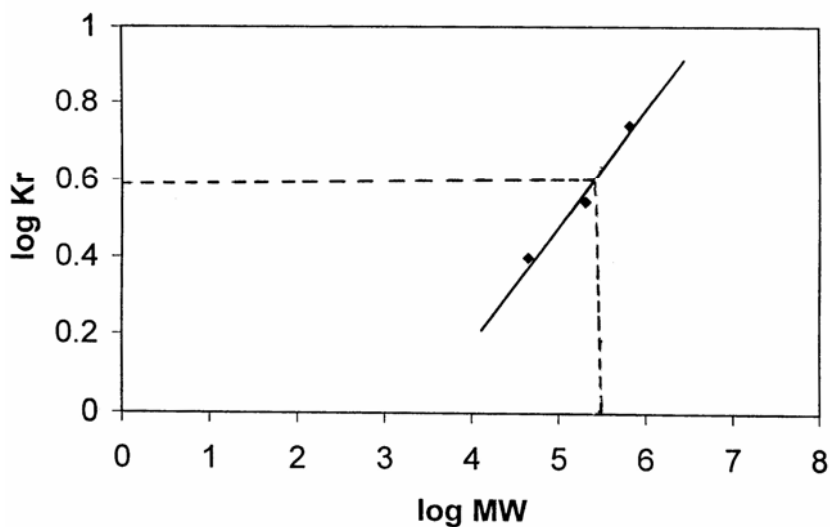


Figure 4. Molecular weight determination of PAP. The data taken from Figure 3 were plotted against M.W. of the markers.

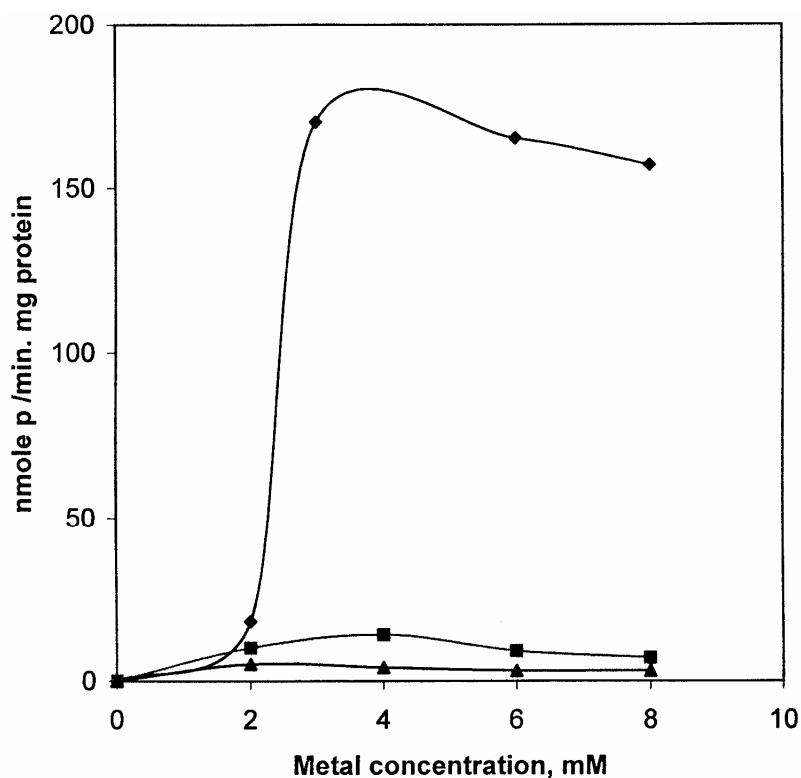


Figure 5. The effects of bivalent cations on PAP activity. The activity of the enzyme was determined in the presence of Mg²⁺ (◆), Ca²⁺ (▲) and Fe²⁺ (■) as described in the Methods.

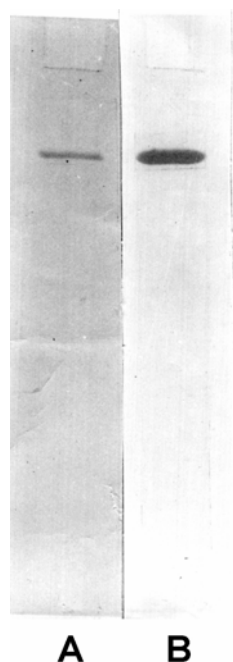


Figure 6. Activity staining of PAP on polyacrylamide gel in the presence of Mg^{2+} and Ca^{2+} . Electrophoresis and activity staining were performed as in Methods. The enzyme (2.4 μ g) was applied on 8% gel concentration. Activity staining was first done in the presence of 5 mM Ca^{2+} (A) and then the gel was incubated at 10 mM Mg^{2+} (B). For detail see text.

Discussion

Structural studies on PAP, a regulatory enzyme in glycerolipid metabolism, have shown contradictory results basically due to the lack of homogeneity of the purified enzyme and the methods employed for these studies [5-9]. In the present work attempts were made to purify cytosolic rat liver PAP to homogeneity for structural studies.

The high M.W. of PAP determined by gel filtration (Fig. 1) seems to be resulted from protein aggregation on Sephadex matrix. Other investigators have reported M.W. of many millions for PAP [5,19]. To overcome this problem, an activity staining procedure was developed in this study to determine PAP M.W. on polyacrylamide gel electrophoresis in the absence of SDS. Activity staining showed only a single active band for the enzyme corresponding to a M.W. of 298 KDa (Figs. 2-4). On SDS-PAGE also only a single band for PAP was observed having M.W. of 47 KDa. The cytosolic liver PAP, therefore, is a hexamer of identical subunits in agreement with a previous report [9].

The effects of bivalent cations on PAP activity have

been contradictory subjects. The present study showed that Ca^{2+} , Cu^{2+} and Fe^{2+} all inhibited the enzyme activity and Mg^{2+} , is required for its activity. In the absence of Mg^{2+} , however, Ca^{2+} up to about 4 mM concentration slightly activated PAP after which the activity declined. Other authors have also reported that Ca^{2+} directly inhibited PAP activity [10] but in the absence of Mg^{2+} exerts stimulatory effect [11]. Butterwith et al. have reported that Zn^{2+} stimulated but Mn^{2+} did not affect PAP activity [5]. Since activity loss of PAP by Ca^{2+} in the gel was restored by Mg^{2+} , it is conceivable that charge density plays a role in enzyme-substrate interaction.

Our recent studies have shown that several bivalent cations up to 1 mM concentration increased PAP activity whereas at higher concentrations exerted inhibitory effects [12]. These studies demonstrate that cation-induced PAP activation is not a result of cation-protein interaction, but is due to the formation of a suitable substratum configuration situated between the lamellar (La) and hexagonal (H4) phases induced by the cations. Although this phase transition could be induced by several cations, Mg^{2+} is probably the only cation capable of generating enzyme-metal-substrate complex.

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