Identification of phosphatidate phosphohydrolase purified from rat liver membranes on SDS-polyacrylamide gel electrophoresis**

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Abstract Phosphatidate phosphohydrolase (PAP; EC 3.1.3.4) insensitive to *N*-ethylmaleimide was partially purified from rat liver membranes by a combination of chromatographic methods, immunabsorption and glycerol gradient centrifugation. The specific activity was increased more than 600-fold over that of the membrane extract. Enzyme antibodies precipitating more than 80% of PAP were obtained and used for the identification of PAP protein on SDS-polyacrylamide gels employing the immunodetection method of Muilerman et al. [(1982) Anal. Biochem. 120, 46–51]. By this approach PAP was localized as a 31 kDa polypeptide.

Key words: Phosphatidate phosphohydrolase; Purification; Antibody; Rat liver

1. Introduction

Phosphatidate phosphohydrolase (PAP; EC 3.1.3.4) catalyses the dephosphorylation of phosphatidate to diacylglycerol, thereby providing hepatocytes both with a substrate for the biosynthesis of triacylglycerol, phosphatidylcholine and phosphatidylethanolamine [1–4] and a second messenger for direct activation of protein kinase C [5–7]. With respect to the latter, interest was focussed on the Mg^{2+}-independent, membraneous form of PAP [8] which seems to control the proportion of phosphatidate and diacylglycerol in the plasma membrane [9]. The importance of PAP is underlined by the multiple activating, signaling and mitogenic effects exerted by phosphatidate both in parenchymal hepatic [10–12] and extrahepatic cells [13,14]. Thus enzymes related to the maintenance of the cellular phosphatidate level deserve great interest. In this study a purification procedure for PAP from rat liver membranes is presented that allowed the generation of enzyme antibodies and for the first time the identification of PAP protein on SDS-polyacrylamide gel electrophoresis. Our results indicate that the polypeptides hitherto regarded as being PAP [15,16] probably do not represent this enzyme.

2. Experimental

2.1. Animals and materials

Male Sprague–Dawley rats and rabbits were purchased from Charles River (Sulzfeld). L-α-Phosphatidic acid, cholate, desoxycholate, sorbitol, glycerol, Tris, *N*-ethylmaleimide (NEM), protease inhibitors, protein A agarose, and iminodiacetic acid Sepharose 6B fast flow, were bought from Sigma (Deisenhofen). Dithiothreitol, Titer-Max and sodium dodecylsulfate (SDS) came from Serva. Ascorbic acid, diethylether, formaldehyde and inorganic chemicals were bought from E. Merck (Darmstadt). Thesit, and standard enzymes were supplied by Boehringer (Mannheim). Acrylamide, bisacrylamide, TEMED, Tween 20, Blotto, and molecular mass standards came from BioRad (Munich). HiLoad 16/60 Superdex 200 prep grade column, and Sephacryl S-400 HR were supplied by Pharmacia (Freiburg).

2.2. Measurement of PAP activity

PAP activity was assayed essentially according to [17]; inorganic phosphate was measured by the method in [18]. Sensitivity against NEM was assayed after preincubation of enzyme fractions in the absence and presence of 5 mM NEM for 15 min at 37°C. Activity measurements were linear with time and protein. The release of 1 μmol of P_i/min is defined as 1 U of PAP activity.

2.3. Purification of PAP

Rats fed ad libitum were stunned and exsanguinized before the liver was removed and placed in ice-cold buffer A (250 mM sorbitol, 40 mM Tris-Cl, 1 mM dithiothreitol, 0.5 mM EDTA, 1 μg/ml each of leupeptin, antipain, chymostatin and pepstatin A, pH 7.8 at 4°C). Further operations were carried out at 4°C unless stated otherwise. Typically 40 livers were homogenized for 1 min in a Waring blender using 3 ml of buffer A per g liver. The homogenate was centrifuged for 10 min at $34,500 \times g_{max}$; the supernatant was further spun for 65 min at $235,000 \times g_{max}$. The pellet obtained was resuspended in a 4-fold volume of buffer A from which sorbitol had been omitted and washed in a Teflon–glass homogenizer. After centrifugation as before, the washing step was 3 times repeated. The final pellet was suspended in the smallest possible volume of buffer B (40 mM Tris-Cl containing 1 mM dithiothreitol, pH 7.8) and freeze-dried. After storage at −20°C for at least 24 h the membrane fraction was stirred at −20°C with precooled diethylether (60 ml per 2.45 g) for 10 min and centrifuged at −20°C for 5 min at $3000 \times g_{max}$. The supernatant was discarded and the pellet treated once more in the same manner before any residual ether was removed in vacuo. For enzyme extraction 2.45 g of the membrane powder was mixed with 50 ml of buffer B and 12 ml of 20% (w/v) sodium cholate solution and homogenized by hand. After standing for 48–72 h, the homogenate was centrifuged at $203,000 \times g_{max}$ for 65 min. The supernatant was removed and kept at −20°C for at least 48 h. After thawing and centrifugation at $203,000 \times g_{max}$ for 125 min the clear supernatant was dialyzed against a 40-fold volume of buffer C (10 mM Tris-Cl, 1 mM dithiothreitol, 0.5 mM EDTA, pH 8.0) for 72 h with one buffer change after 24 h.

2.3.1. First DEAE cellulose treatment. Moist Whatman DE 52 cellulose equilibrated with buffer C was added to the dialysate (100 mg/ml) and kept in an ice-bath for 15 min with occasional stirring. The PAP containing supernatant was collected by filtration and the cellulose was washed once with buffer C with about 10% of the starting volume.

2.3.2. Precipitation of PAP at pH 5.3. To the combined filtrates 10% acetic acid was added dropwise until the pH was lowered to 5.3, resulting in a marked turbidity. After centrifugation at $16,000 \times g_{max}$ for 10 min the clear, inactive supernatant, was discarded; the brownish pellet was dissolved with about 10% of the original volume using buffer D (10 mM Tris-Cl, 1 mM dithiothreitol, 0.5 mM EDTA, 100 mM NaCl, 0.5% cholate, pH 8.0) by increasing final cholate concentration to 1.5%, and pH adjustment with solid $KHCO_3$, before the enzyme extract was kept at −20°C for at least 48 h. After thawing Pefabloc was added to give a final concentration of 0.5 mM followed by centrifugation at $235,000 \times g_{max}$ for 1 h. The supernatant was filtered (0.22 μm) to remove any turbidity.

2.3.3. Metal chelate affinity chromatography. Typically 200 ml of the clear, red-brown enzyme solution was applied on top of an imi-

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Table 1
Summary of PAP purification from rat liver by acid precipitation and chromatographic steps[a]

Purification step	PAP activity (mU)	Protein (mg)	Specific activity (mU/mg)	Yield (%)	Purification factor
Dialyzedextract	20,427	4952	4.1	100	1
DEAE (1st)	18,768	3197	5.9	92	1.4
pH 5.3	19,590	1331	14.7	96	3.6
Ni^{2+}-column	4034	43.6	92.5	20	22.6
DEAE (2nd)	2169	5.0	434	11	106
Gel filtration	571	0.21	2719	2.8	663

[a]Immunopurification and glycerol gradient centrifugation had not been included for the reasons given in the text.

nodiacetic acid column (5×12 cm) loaded with Ni^{2+}, and equilibrated with 10 mM Tris-Cl buffer, pH 8.0, containing 100 mM NaCl. After the enzyme had entered the column, washing was started with 2 column volumes of equilibration buffer followed by 4.5 volumes of equilibration buffer containing 0.2% Thesit, 8 column volumes of 20 mM Na-acetate buffer, pH 6.0, containing 100 mM NaCl and 0.2% Thesit, and 7 column volumes of this buffer mixture at pH 5.6. For the elution of the enzyme the pH of this buffer was lowered to 5.2. Fractions of increased specific activity were pooled, concentrated about 15-fold by ultrafiltration and dialyzed against a 50-fold volume of buffer C for 3 h and a 150-fold volume overnight. Any precipitate formed was removed by centrifugation and Pefabloc was added to 0.5 mM.

2.3.4. Second DEAE cellulose treatment. DE 52 cellulose equilibrated with 10 mM Tris-Cl buffer, pH 8.0, containing 1 mM dithiothreitol, 0.5 mM EDTA and 0.2% Thesit was added to the enzyme solution (1.2 g of moist cellulose per mg of protein), stirred and collected by centrifugation. The cellulose was washed 3-times with a 2-fold volume of equilibration buffer before the enzyme was eluted with equilibration buffer to which 40 mM NaCl and 0.02% Thesit had been added. Typically 10 washing steps were performed, each buffer volume corresponding to the starting cellulose volume.

2.3.5. Gel filtration. The pooled DE 52-eluates were concentrated about 100-fold by ultrafiltration and applied on top of a Sephacryl S-400 column (2.6×95 cm) equilibrated and eluted with buffer D. Alternatively gel filtration of 0.8 ml aliquots was performed on a Superdex column (1.6×60 cm) in a Pharmacia FPLC system. Fractions of 1 ml were collected at a flow rate of 0.5 ml/min; enzyme elution culminated at 56 ml.

2.3.6. Immunopurification. The fractions containing the peak of PAP activity were concentrated about 30-fold by ultrafiltration and diluted at least 1:3 with buffer E (20 mM Tris-Cl, 100 mM NaCl, 1 mM EDTA, 0.1% Thesit, 0.02% NaN_3, pH 8.0). The presence of Thesit prevented the sticking of the enzyme to the subsequently added protein A agarose. The antisera were raised as described below and used as a mixture from the different rabbits. For the determination of the appropriate binding conditions 25–250 µl of a 1:4 protein A agarose suspension in buffer E coupled (see below) to preimmune and immune globulins, respectively, was centrifuged and the gel pellet washed once with buffer E. After discarding the supernatant, 100 µl of appropriately diluted enzyme solution was added and the gel suspension shaken overnight. The gel was separated by brief centrifugation and the supernatant was analyzed both for enzyme activity and protein pattern by SDS gel electrophoresis. The optimal proportion resulting from these pilot experiments was scaled up for the treatment of the bulk of the preparation.

2.3.7. Glycerol gradient centrifugation. The immunopurified enzyme was concentrated with Centricon (Amicon) before 0.1 ml was layered on a linear 10–30% (w/w) glycerol gradient in buffer D and centrifuged at 38,500 rpm in a Beckman SW50.1 rotor for 17 h. Fractions of 180 µl were collected from the punctured tube. The peak of activity eluted at 2.1 ml.

2.4. Antibody production and coupling to protein A agarose

Polyclonal antibodies were raised in rabbits by repeated subcutaneous injections of protein (20–200 µg) TiterMax adjuvant emulsions. The antigens used for immunization were protein mixtures obtained at the various stages of the purification procedure. One animal was injected with the concentrated washing solutions collected after binding of the enzyme activity to the metal affinity column, a second rabbit received a PAP-devoid supernatant concentrate after PAP-binding to DE 52 cellulose, a third one was immunized against the proteins contained in the fractions just following PAP elution from the gel filtration column. Additional animals were injected with the peak fractions of Superdex chromatography and glycerol gradient centrifugation, respectively. Coupling of immunoglobulins in antisera and preimmunsera, respectively, to protein A agarose was carried out according to [19].

2.5. Gel electrophoresis

Electrophoresis under denaturing conditions was carried out on 10% polyacrylamide gels (0.75 mm) as described by Laemmli [20] using a Pharmacia Midget system. Proteins were stained with silver [21].

2.6. Identification of PAP on SDS gel

The immunotechnique of Muilerman et al. [22] was adapted for the detection of inactive PAP-protein. After SDS-polyacrylamide gel electrophoresis, protein was transferred to 0.2 µM nitrocellulose for 30 min at 8 V using transfer buffer I of [23]. Strips were cut and individually incubated for 1 h at room temperature with 5% Blotto in Tris-buffered saline–Tween 20 (TBST) as detailed in [23] to saturate remaining protein binding sites. After washing with TBST (two times with 5 ml, 5 min each), the strips were incubated overnight in preimmunserum and antiserum, respectively, raised against PAP after glycerol gradient fractionation, diluted 1:5 with TBST. Washing with 5 ml of buffer F (50 mM Tris-Cl, pH 7.4, 0.5 M NaCl, 0.2% Thesit, 0.5% desoxycholate and 0.1% SDS) followed (three times, 30 min each). After further washing with TBST (two times, 5 min each) incubation was continued with 1.5 ml/strip of native PAP in TBST

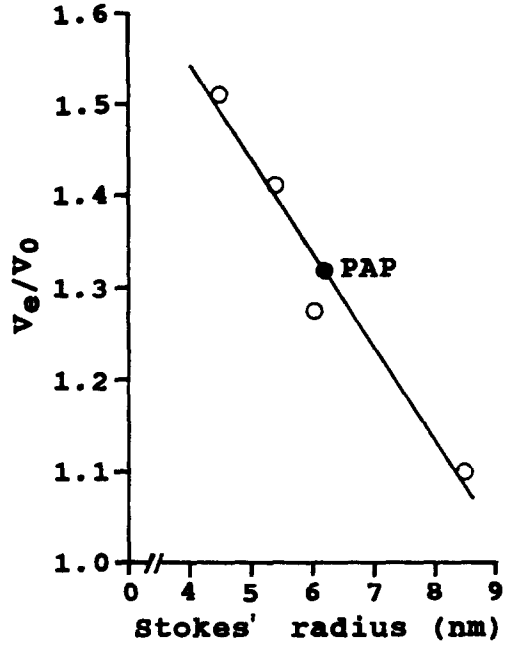

Fig. 1. Estimation of the Stokes' radius of PAP. For gel filtration on Superdex see section 2.3.5. The data for the standard proteins thyroglobulin (8.5 nm), apoferritin (6.1 nm), pyruvate kinase (5.4 nm) and aldolase (4.5 nm) were taken from [24].

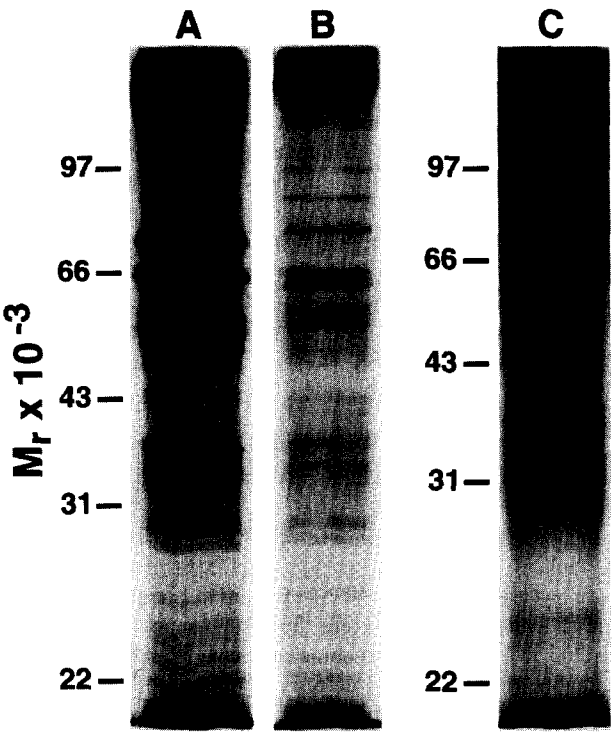

Fig. 2. Staining pattern of the 40 mM NaCl DEAE-eluate after immunabsorption (A,B) and glycerol gradient centrifugation (C). Immunopurification was performed as detailed in section 2.3.6, except that the PAP preparation after the second DEAE-cellulose treatment was employed. The patterns obtained after incubation with preimmune (A) and immune (B) globulins are shown. (C) The peak fraction of a glycerol gradient (section 2.3.7) was dialyzed against 10 mM ammonium bicarbonate, vacuum-dried and dissolved in loading buffer prior to electrophoresis (section 2.5).

(corresponding to 9 mU) for 12–16 h. The strips were then washed once with 5 ml buffer F for 20 min, five times with TBST (20 min each) and cut into 2–5 mm sections which were incubated in 0.3 ml of 0.66 mM phosphatidate in 0.2 M Tris-Cl/0.15% Tween 20, pH 7.0, at 25°C for 12–16 h before phosphate was determined.

2.7. Protein estimation

Protein concentration was determined using the BCA assay (Pierce) and bovine serum albumin as a standard.

3. Results

The present study combines both chromatographic and immunological methods for the purification of PAP from rat liver membranes. The enzyme activity was found to be Mg^{2+}-independent, NEM-insensitive and inhibited by NaF and metal (Zn^{2+}, Mn^{2+}) ions (data not shown) in agreement with the findings of others [15]. Metal affinity, DEAE-cellulose and gel chromatography resulted in a 600-fold increase in specific activity over that of the crude membrane fraction (Table 1). Further purification was effected by immunabsorption and glycerol gradient centrifugation; the calculation of the respective purification factors, however, was hampered by the fact that the amount of protein was too low for accurate measurement and by the marked loss of activity during protein (and concomitant detergent) concentration. Therefore, the purification factors obtained for each of these steps of about 2 may be underestimated. The purification procedure worked highly reproducible. Cholate solubilized more than

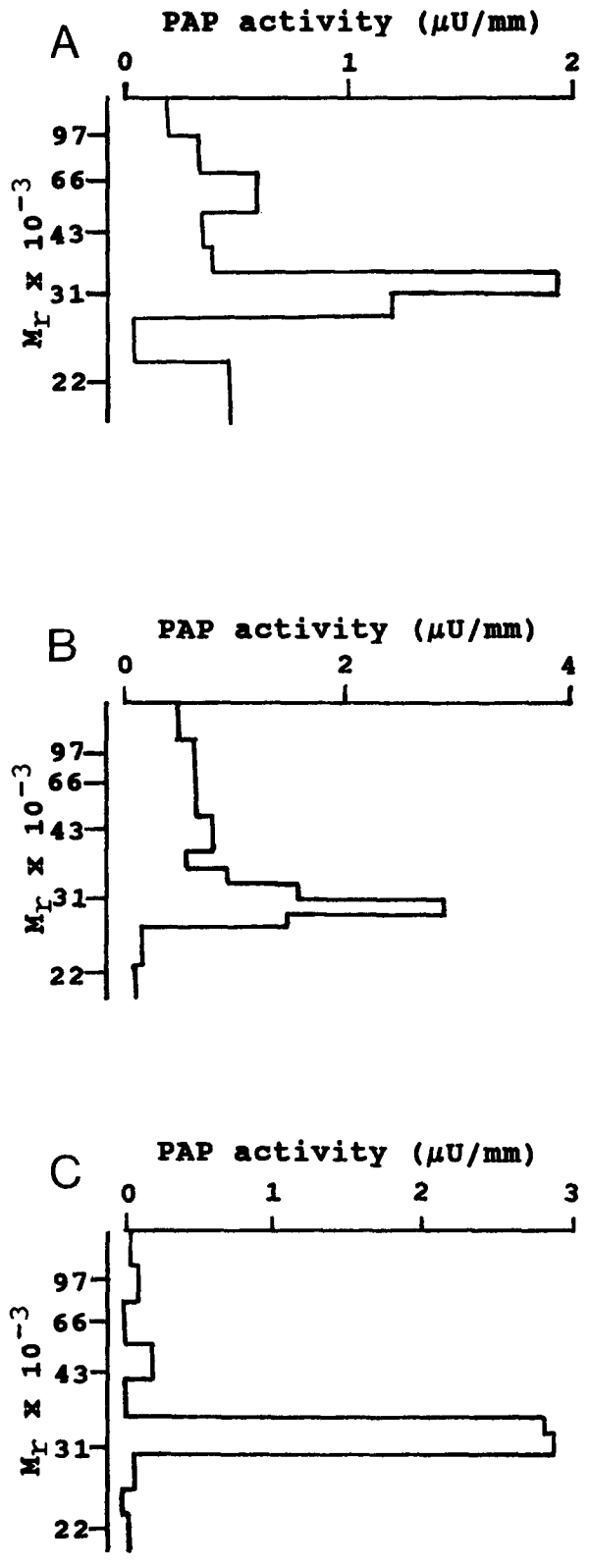

Fig. 3. Identification of PAP after SDS-polyacrylamide gel electrophoresis. Lanes containing 0.3 μg of protein after immunopurification (A), 74 μg of protein after purification up to step 3 (B) and 16 μg of protein of the 250 mM NaCl eluate from DEAE-cellulose after Superdex chromatography were processed as stated in section 2.6. Positions of phosphorylase *b* (M_r 97 kDa), bovine serum albumin (M_r 66 kDa), ovalbumin (M_r 43 kDa) carbonic anhydrase (M_r 31 kDa) and trypsin inhibitor (M_r 22 kDa) on a parallel lane are indicated. The PAP activities shown were corrected for the effect of the preimmunserum.

90% of total membrane activity. Although the first DEAE-step only moderately increased specific activity, it improved overall reproducibility. Regarding the enzyme precipitation at pH 5.3, the prior removal of cholate by thorough dialysis was essential for complete enzyme recovery. The increase in specific activity after acid precipitation was due to the removal of a bulky, inactive pellet after freezing and centrifugation. In metal affinity purification little binding of PAP to the column occurred in the absence of cholate whereas in its presence binding was almost quantitative. During this step the replacement of cholate by the non-ionic detergent Thesit was necessary for enzyme elution at pH 5.2. The enzyme activity at this pH was stable for at least 48 h. Enzyme recovery before ultrafiltration and pH adjustment was some 50%. The pH was brought to 8.0 by dialysis, as pH elevation by the addition of bicarbonate or NaOH abolished enzyme activity. For the following DEAE-step the presence of Thesit both for binding and elution was necessary. The batchwise performance was not inferior to DEAE column chromatography with respect to both yield and specific activity. After completion of enzyme elution at 40 mM NaCl, some 25% of the enzyme activity applied were recovered by increasing NaCl concentration to 250 mM; the specific activity of this portion, however, was one order of magnitude lower. Gel filtration of the 40 mM NaCl DEAE-eluate resulted in a single peak of activity. From its elution behaviour the Stokes' radius of PAP was estimated [24] to be 6.1 nm (Fig. 1). As no substantial progress in enzyme purification could be achieved by a variety of chromatographic methods, isoelectric focussing and native polyacrylamide gel electrophoresis, further purification was attempted by the use of antibodies against contaminating proteins. These were raised against PAP-devoid fractions as obtained at the various stages of purification, linked to protein A agarose and used as a tool for immunopurification of the pooled enzyme fractions after gel filtration. Analysis of the supernatant of this immunoprecipitation step by SDS-gel electrophoresis revealed that the antibodies, as compared with the preimmune control (Fig. 2A) strikingly diminished the bands (Fig. 2B). As some 90% of the PAP activity were recovered in the supernatant, these results clearly demonstrate that PAP was not associated with the prominent 50–60, 66 and 76 kDa bands (Fig. 2A). To get information on PAP localization within the spectrum of the remaining proteins, the supernatant was concentrated and subjected to glycerol gradient centrifugation as it offered the possibility to correlate enzyme activity of the gradient fractions with the protein pattern on SDS-gels. PAP activity sedimented as a nearly symmetrical peak, yielding a sedimentation coefficient of 7.3S as compared with catalase (11.3S), aldolase (7.3S), bovine serum albumin (4.3S) and ovalbumin (3.6S) run in a parallel glycerol gradient as the standards. PAP localization in this way, however, was complicated by the fact that three bands corresponding to 38, 34 and 31 kDa, as shown in Fig. 2C for the peak fraction appeared to follow enzyme distribution over the gradient, whereas the staining intensity of the residual gel changed independent of PAP activity. Therefore, PAP identification was approached by the immunodetection method of Muilerman et al. [22]. Accordingly, denatured enzyme protein on nitrocellulose is incubated with excess antibodies to form complexes with one antigen binding site remaining free; on the subsequent incubation with native enzyme a 'sandwich' of (denatured)enzyme/immunoglobulin/(active)enzyme is generated, which is detectable by product formation from enzyme substrate. Antibodies for this procedure obtained by immunization with PAP after glycerol gradient centrifugation precipitated more than 80% of PAP activity, whereas a much weaker effect resulted from immunization with PAP after gel filtration (data not shown). Fig. 3 summarizes the results found with the immunodetection technique of Muilerman et al. [22]. Obviously, the peak of PAP is located close to the 31 kDa marker carbonic anhydrase. This finding applies for both the highly purified enzyme after immunopurification (Fig. 3A) and the enzyme preparation at an early stage (step 3) of purification (Fig. 3B) and the enzyme in the 250 mM NaCl DEAE-eluate as well (Fig. 3C).

4. Discussion

Despite considerable efforts over decades on purification of PAP from a mammalian source [17,25,26] very little is known about structure and regulation of PAP. This is mainly due to the lack of pure enzyme preparations both of the NEM-sensitive cytosolic and the NEM-insensitive membraneous PAP forms. Kanoh et al. [27] were the first to report on PAP from porcine thymus membranes as a single M_r 83 kDa protein band on SDS-gel electrophoresis. Fleming and Yeaman, applying the method of Kanoh et al. [27] to rat liver membranes, also reported on a 83 kDa polypeptide, which was considered, but not documented to represent PAP; in addition these authors found essential PAP activity in a mixture of proteins ranging from 30 to 83 kDa [15]. With regard to the 83 kDa protein the recent statement from Kanoh's laboratory is noteworthy that a 35 kDa rather than the 83 kDa polypeptide could account for the PAP activity in their enzyme preparation [28]. By ion exchange and hydroxylapatite chromatographic purification of PAP from rat liver membranes Waggoner et al. distinguished between anionic and cationic enzyme forms of 53 and 51 kDa, respectively, [16]. The purification factor achieved by these authors of about 270-fold over the membrane extract [16] is comparable with that of our procedure prior to immunopurification (Table 1). Their claim [16] of having purified cationic PAP to homogeneity is in contrast to the SDS-gel pattern presented, which shows prominent bands in the 50–60 kDa region and a number of weaker bands in the range of about 27–120 kDa. Waggoner et al. immunizing with this protein mixture produced antibodies that precipitated about 50% of PAP activity and stained the 50–60 kDa region in Western blots [16]. From these findings the authors concluded that the 51 and 53 kDa polypeptides represent PAP [16]. However, no evidence had been provided for a link between the results of PAP immunoprecipitation and Western blot analysis. Moreover, the possibility remains that PAP was not directly precipitated by PAP antibodies, but rather indirectly by immunoprecipitation of the abundant 50–60 kDa species. In contrast, the immunological approach of the present study directly signals the location of PAP protein by enzyme product formation. Fig. 3 clearly shows that this occurred at the 31 kDa site, with little, if any, phosphate release in the 50–60 kDa interval. It seems reasonable, therefore, to assume that PAP contains (a) 31 kDa subunit(s). From the Stokes' radius (Fig. 1) and the sedimentation coefficient of PAP of 7.3S a molecular mass for the native enzyme of 186 kDa can be derived [24]. Thus, it is tempting to speculate that PAP may display a hexameric subunit structure. Peptide se-

quencing may help via cDNA cloning to answer this and other questions as to the nature of PAP. The peptide sequences so far derived from the 31 kDa band are different from the databank contents on the as yet identified polypeptides.

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