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Short Communication

Purification of Mg^{2+}-dependent phosphatidate phosphohydrolase from rat liver: new steps and aspects*

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Abstract

A new procedure for the partial purification of Mg^{2+}-dependent, *N*-ethylmaleimide-sensitive phosphatidate phosphohydrolase (Mg^{2+}-PAP; EC 3.1.3.4) from rat liver cytosol is described, using protein precipitation with $MgCl_2$, gel filtration on Sephacryl S-400, chromatography on DEAE-cellulose and affinity chromatography on calmodulin-agarose. From the parallel change in staining intensity and in the level of the specific activity of enzyme fractions, a relationship between a 90-kDa SDS gel band, identified as the β-isoform of the 90-kDa heat shock protein, and Mg^{2+}-PAP could be detected.

Keywords: 90-kDa heat shock protein β-isoform; purification; rat liver cytosol.

Among the enzymes cooperating in hepatic triacylglycerol and phospholipid synthesis (Coleman et al., 2000), Mg^{2+}-dependent, *N*-ethylmaleimide-sensitive phosphatidate phosphohydrolase (Mg^{2+}-PAP; EC 3.1.3.4) is perhaps the least known. This is in striking contrast to the immense pathophysiological impact of disturbed lipid production and points to the great difficulties impeding the purification of this enzyme. Two decades after the report on the hitherto most successful partial purification from rat liver cytosol (Butterwith et al., 1984), we describe our efforts to gain more insight into the nature of this enzyme protein, resulting in new purification steps that lead to the detection of a possible relation between Mg^{2+}-stimulated phosphatidate hydrolysis and the 90-kDa heat shock protein β-isoform (HSP90β). The purification of Mg^{2+}-PAP is hampered by the fact that the enzyme is firmly associated with a number of proteins. Moreover, after initial purification, the enzyme activity becomes rather labile, with losses amounting up to 50% within 24 h. The step first reported (Hosaka et al., 1975) to yield an appreciable increase in the specific activity of Mg^{2+}-PAP is binding to calcium phosphate gel and elution with phosphate buffer. Apart from the fact that this procedure complicates the Mg^{2+}-PAP assay, we repeatedly noted irreversible enzyme binding to commercial hydroxyapatite preparations. Here we describe new, simple and highly reproducible steps for Mg^{2+}-PAP purification. The first step takes advantage of the observation that the addition of $MgCl_2$ to an appropriately dialysed particle-free supernatant causes almost complete precipitation of the Mg^{2+}-PAP activity. From this precipitate, up to 80% of Mg^{2+}-PAP activity could be extracted by KSCN treatment. Under the Tris, pH 7.2 conditions described, some 90% of the contaminating proteins remained in the pellet. These data, as well as our respective SDS gel patterns (not shown), warrant the notion that the use of magnesium chloride is superior to ammonium sulfate (Hosaka et al., 1975) in enzyme preparation. The basis of the second effective purification step is filtration of the KSCN extract on Sephacryl S-400 in the presence of Nonidet P-40. This was also performed after pretreatment of the KSCN extract with dimethylmaleic anhydride (DMMA), because DMMA favours the dissociation of protein complexes in a reversible, pH-regulated manner (Wieland et al., 1979). In our functional tests, DMMA led to ca. 90% loss of Mg^{2+}-PAP activity, which could be recovered by acidification by some 50%, and thus we were interested in the behaviour of the freshly inactivated KSCN extract on Sephacryl S-400. DMMA apparently caused dissociation of Mg^{2+}-PAP from associated proteins, as only one activity peak (elution volume corresponding to approx. 550 kDa) was observed, whereas in the control (acetone alone) sample an additional peak was found at approximately 1500 kDa, which amounted to ~30% of total activity, and much lower specific activity (factor of seven; data not shown). The steps of KSCN extraction, gel filtration and calmodulin-agarose chromatography each yielded 8–10-fold purification (Table 1). Chromatography on other media such as DEAE-cellulose and heparin-agarose was less effective, resulting in purification factors of 2–3. Unfortunately, the use of known affinity chromatography media such as 2′,5′-ADP-, 5′-AMP-, and ATP-agaroses to separate the $NAD(P)^+$- and ATP-binding proteins from Mg^{2+}-PAP failed to give satisfactory results. The same was found for thiol-, octyl-, phenyl-, and polylysine-Sepharoses, as well as sulfoethyl- and carboxymethyl-celluloses, when tested under a variety of conditions. Moreover, native PAGE on gels down to 2% (Peacock and Dingman, 1968) was unsuccessful, as the protein complex did not enter the gels to any significant extent: the Mg^{2+}-PAP activity was detectable in the top 2-mm section of the gel only. Subsequent analysis of the section under denaturing conditions (Laemmli, 1970) displayed a great number of bands, from which no trace of Mg^{2+}-PAP activity could be recovered when tested after SDS removal, singly or in combination.

*Dedicated to Professor O.H. Wieland on the occasion of his 85th birthday.

Table 1 Purification steps and their respective purification factors.

Step	Increase in specific activity (A)
Precipitation of liver cytosol (B) with $MgCl_2$ (C)	8–10-fold
Sephacryl S-400 gel filtration (D)	8–10-fold
DEAE-cellulose chromatography (E)	2–3-fold
Calmodulin-agarose chromatography (F)	8–10-fold

(A) For measurement of Mg^{2+}-PAP activity, a phosphatidate (Sigma, Deisenhofen, Germany) stock solution (PS) was prepared by dissolving 1.4 mg of phosphatidic acid per ml of 0.2 M Tris-HCl buffer containing 0.15% (v/v) Tween 20, pH 7.5 at 37°C (TTB). The assay mixture contained 100 µl of PS, 10 µl of 0.1 M $MgCl_2$, or water (control), 5 µl of 13.2 mM EDTA (pH 8.0), 5 µl of 13.2 mM EGTA (pH 7.5), and 35 µl TTB before the reaction was started by the addition of 100 µl of the sample. Incubation was performed at 37°C for 15–60 min before 10 µl of 70% $HClO_4$ was added and the mixture was chilled on ice. After centrifugation for 2 min at 12 000 g_{max}, inorganic phosphate in the clear supernatant was determined according to Mavis et al. (1978). The release of 1 µmol of P_i/min at 37°C due to the presence of Mg^{2+} is defined as 1 U of Mg^{2+}-PAP activity. Protein concentration was determined by UV absorption at 280 nm or using commercial kits.

(B) For liver cytosol preparation, rats were stunned and exsanguinated before the liver was removed and placed in ice-cold buffer A [250 mM sorbitol, 40 mM Tris-HCl, 1 mM dithiothreitol (DTT), 0.5 mM EDTA, 1 µg/ml each of antipain, chymostatin, leupeptin and pepstatin A, pH 7.8 at 4°C]. Further operations were carried out at 4°C unless stated otherwise. Typically, 40 livers were homogenised for 1 min in a Waring blender using 3 ml of buffer A per 1 g of liver. The homogenate was centrifuged for 45 min at 34 500 g_{max}; the supernatant was transferred (omitting loosely packed material) to Beckman quick-seal tubes and centrifuged for 60 min at 235 000 g_{max}. The supernatant was filtered through cheesecloth and stored in 40-ml aliquots at -20°C.

(C) Usually six aliquots (240 ml) were thawed overnight and centrifuged for 10 min at 27 000 g_{max}. The supernatant was dialysed against a 12.5-fold volume of buffer B (10 mM Tris-HCl, 1 mM DTT, 0.5 mM EDTA, pH 7.2) at 4°C for 4 h before the buffer was changed and dialysis was continued overnight. Any turbidity was removed by centrifugation. Without delay, the red supernatant was placed in a glass beaker and 0.1 M $MgCl_2$ amounting to 1/10 of the volume of the supernatant was added under stirring. The beaker was placed in a 33°C water bath and the content brought to 25°C; stirring was continued for 10 min, before the turbid suspension was cooled to 10°C and centrifuged at 4°C for 15 min at 27 000 g_{max}. The clear supernatant was removed and the pellet was washed once with buffer B. After centrifugation as above, the pellet was resuspended in half the original volume of buffer B and homogenised by hand in a glass-Teflon homogeniser, before 50% the original volume of 2 M KSCN solution in buffer B was added under stirring. After clearing for some 30–45 min, the slightly turbid extract was dialysed against a 15-fold volume of buffer B. After 3 h the buffer was renewed and dialysis was continued overnight, resulting in a considerable amount of insoluble material, which was removed by centrifugation as above. The supernatant was further dialysed against a 15-fold volume of buffer C (10 mM HEPES, 0.2 mM DTT, pH 8.2) at 4°C for 2 days, with three changes. After centrifugation, a yellowish, faintly opalescent supernatant (KSCN extract) was obtained.

(D) Prior to gel filtration, the KSCN extract was concentrated by acid precipitation: approximately 42 µl of 1% acetic acid was added stepwise per 1 ml of extract until the pH electrode showed a value of 6.1. The precipitated protein was collected by centrifugation; the supernatant was completely removed and the pellet was suspended in 7% of the original volume of buffer D (100 mM HEPES, 2 mM DTT, 0.5% Nonidet P-40, pH 8.2) and homogenised by hand in a Potter homogeniser. Finally, 3% of the original volume of buffer E (10 mM HEPES, 0.2 mM DTT, 0.5% Nonidet P-40, pH 8.2) was added and kept on ice for 30 min, before a clear yellow extract was obtained by centrifugation as above; this was applied at 30 ml/h to the top of a Sephacryl S-400 column (5×91 cm) equilibrated with buffer E and eluted with the same buffer at 60 ml/h.

(E) Combined fractions after gel filtration were concentrated by acid precipitation (see above) and loaded onto a 10-ml column of DE 52 cellulose equilibrated with buffer E. The column was washed with buffer E containing 0.2 M NaCl, before the enzyme activity was eluted with buffer E containing 0.32 M NaCl.

(F) A column (1.2×9 cm) of calmodulin-agarose (Sigma) was equilibrated with buffer E containing 50 mM NaCl and 50 µM $CaCl_2$. Enzyme samples were fortified with 50 mM NaCl and 100 µM $CaCl_2$ prior to application. Contaminating proteins were removed by washing with three column volumes of equilibration buffer, followed by three column volumes of buffer E containing 0.3 M NaCl and 50 µM $CaCl_2$ and one volume of buffer E with 0.3 M NaCl. Enzyme activity was eluted with the latter buffer, to which 0.16 ml of a 0.25 M stock solution of EGTA (pH 7.5 with NaOH) per 100 ml had been added.

Regarding enzyme stability, the activity of the KSCN extract decreased only slowly within 3–4 weeks at 4°C; however, further purification markedly destabilised the enzyme activity. One possible reason might be the separation of a stabilising component(s) from Mg^{2+}-PAP during the purification procedure. In this context, it seems noteworthy that a 34-kDa band identified by amino acid sequencing as apolipoprotein E lacking the signal peptide (DeMattos et al., 1999) was markedly diminished on further purification of the KSCN extract, as the involvement of apolipoprotein E in hepatic triglyceride synthesis has been documented (Huang et al., 1998; Mensenkamp

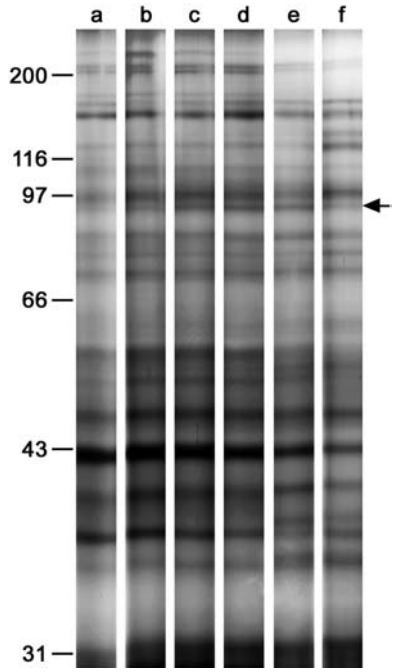

Figure 1 Pattern of protein bands of Sephacryl S-400 fractions subjected to 10% SDS gel electrophoresis.
Routinely, 7.5 μg protein/lane was subjected to SDS gel (1.5 mm) electrophoresis as described by Laemmli (1970). Sample protein concentrations were adjusted to 1 mg/ml by trichloroacetic acid (TCA) precipitation of aliquots, using 316 μl of ice-cold 25% TCA per 1 ml of sample. After a minimum precipitation time of 2 h at 4°C, samples were centrifuged in the cold in an Eppendorf centrifuge at full speed for 20 min and the pellets were washed twice with 1 ml of prechilled (-20°C) acetone. Each washing was centrifuged for 15 min as above. Following the complete removal of acetone, the precipitate was dissolved overnight in reducing Roti-Load (Roth, Karlsruhe, Germany) and heated for 1 min at 95°C prior to electrophoresis. Gels were stained with silver (Blum et al., 1987) and dried as described by Samal (1987), except for gels intended for protein analysis by amino acid sequencing and peptide mass spectrometry, which were stained with Coomassie Brilliant Blue prior to cutting out the band(s) of interest from the wet gel. The staining intensity of a 90-kDa band (arrow) in lanes a–f corresponds to the specific activity of the enzyme fractions used, amounting to 6, 11, 21, 24, 18 and 10 mU/mg protein, respectively. Molecular mass markers are indicated in kDa on the left.

et al., 1999). However, our recombination experiments failed to improve the enzyme stability. Thus, for this marked increase in instability concomitant with the increase in specific activity, the activity of the overall enzyme purification should be estimated from the factors yielded from step to step, rather than from the final absolute value of the specific activity to avoid gross underestimation. Compared on this basis, our purification procedure seems to be at least as effective as that of others who reported a 416-fold purification (Butterwith et al., 1984).

According to Butterwith et al. (1984), their preparations displayed more than 10 bands in SDS gel that were not assigned to enzyme activity. For our preparations, assignment was on the basis that the staining intensity of the protein bands parallels the level of specific activity measured in the native state prior to electrophoresis. The application of a constant amount of protein to each lane was therefore prerequisite. This was achieved by carrying (different) volumes of purified fractions through a protein-precipitation procedure and dissolving their content with loading buffer to a uniform protein concentration of 1 mg/ml. On this basis, the change in band intensity proportional to the relative amount of Mg^{2+}-PAP protein per lane was screened in non-gradient gels of 5–15% polyacrylamide, run with a variety of protein loads, to determine the appropriate visualisation conditions for band detection and resolution. Figure 1 shows that in the Sephacryl S-400 fractions a band in the 90-kDa region best fitted the above criterion. This band alone was intensified by further purification (Figure 2) in accord with the increase in specific activity. A 6.5% gel allowed further resolution, displaying two narrow bands of apparent mass of 93 and 90 kDa (Figure 3, lane a). These were identified by mass spectrometry of the tryptic peptides as the α- and β-isoforms, respectively, of the 90-kDa heat shock protein. Calmodulin-agarose chromatography revealed that Mg^{2+}-PAP activity was increased in the

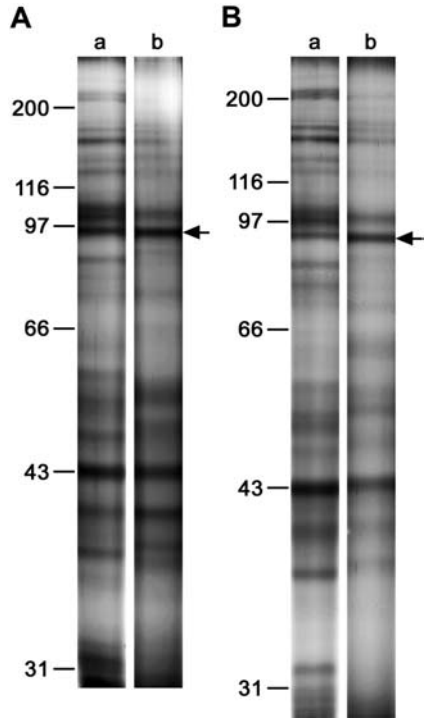

Figure 2 Pattern of protein bands on 10% SDS gels of the KSCN extract after control (A) and DMMA (B) incubation, respectively.
Samples were purified on Sephacryl S-400 and DEAE-cellulose (a) and further on calmodulin-agarose (b) as described in the legend to Table 1. The 90-kDa band (arrow) is the only one intensified, in accord with the increase in specific activity. For reversible inactivation by DMMA (ICN, Eschwege, Germany) the HEPES concentration of the enzyme solution was increased to 50 mM before an appropriate amount of a stock solution (100 mg/ml) of DMMA freshly dissolved in acetone, or acetone alone (control), was added to give a final concentration of 7 mM DMMA. Incubation was performed at 4°C for 30 min, leading to Mg^{2+}-PAP inactivation; for its reactivation, the sample protein was precipitated by the addition of 1% acetic acid to give pH 5.6 (glass electrode). After centrifugation and complete removal of the supernatant, the pellet was redissolved in 80 mM HEPES buffer, pH 8.2. Spontaneous reactivation (without acidification) occurred during storage overnight in a cold room.

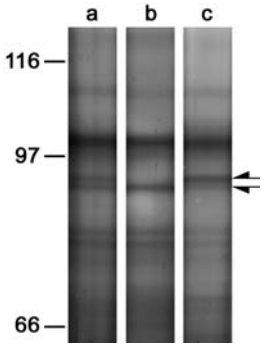

Figure 3 Resolution of the 90-kDa band into two bands (arrows) by 6.5% SDS gel electrophoresis.
The relevant portion of the gel shows the patterns of the KSCN extract purified by Sephacryl S-400 and DEAE-cellulose chromatography before (a) and after calmodulin-agarose chromatography (b,c). Mg^{2+}-PAP activity was increased in the EGTA eluate (b) and diminished in the flow-through (c). Molecular mass markers in kDa are indicated on the left.

fractions containing HSP90β (Figure 3, lane b) and diminished in the column flow-through, which contained mostly the α-form (Figure 3, lane c). From this result, it appears reasonable to conclude that HSP90β might have a functional relation to Mg^{2+}-PAP. In retrospect, this helps to explain phenomena observed during the purification of Mg^{2+}-PAP on the basis of known properties of HSP90β, such as its ability to bind to calmodulin (Koyasu et al., 1986; Minami et al., 1993), to form large protein aggregates not entering a polyacrylamide gel under native conditions (Nemoto and Sato, 1998) and to bind to actin under conditions of Mg^{2+} concentration and temperature (Koyasu et al., 1986; Nishida et al., 1986) very similar to those used in this study for the precipitation of Mg^{2+}-PAP from the particle-free cytosolic supernatant.

The results presented here not only provide new steps for purification of the enzyme, but also open new aspects for the understanding of previous findings, such as the translocation of Mg^{2+}-PAP from the cytosolic to the microsomal compartment (Cascales et al., 1984; Martín-Sanz et al., 1984; Hopewell et al., 1985) and the effect of calcium on Mg^{2+}-PAP activity (Pollard and Brindley, 1984; Martin et al., 1986). According to the evidence presented, it may well be that the (direct or indirect) relationship of $Mg2^{+}$-PAP activity to HSP90β represents a common basis to characterise these phenomena as actin/calmodulin-dependent interactions.

Mg^{2+}-PAP is considered an important regulatory enzyme in hepatic lipid synthesis (Coleman et al., 2000). Any progress in its exploration, which stagnated in recent years, is therefore of value, as it contributes to better understanding of a (patho)physiologically very important field.

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