On the 6-phosphofructo-1-kinase phosphatase activity of protein phosphatase 2C and its dimeric nature

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A recently described 6-phosphofructo-1-kinase phosphatase (PFK-phospatase [1]) shared several properties with protein phosphatase 2C [2,3], but exhibited differences with respect to molecular mass and substrate specificity. Chromatography on histone-Sepharose, gel filtration experiments on Sephacryl S-200 and Sephadex G-100 as well as sucrose density gradient centrifugation [4] show that both enzyme preparations behave identically under all experimental conditions used. The low activity of PFK-phosphatase phosphorylated histone H2B [1] had resulted from an inhibition of the enzyme by high concentrations of this substrate. The apparent molecular mass of protein phosphatase 2C as calculated from Sephacryl chromatography and sedimentation analysis is about 90 kDa, the molecular mass obtained by SDS gel electrophoresis about 45 kDa. The native enzyme therefore seems to be a dimer consisting probably of 2 identical subunits. Accordingly, the previously described PFK-phosphatase is protein phosphatase 2C.

Protein phosphatase *6-Phosphofructo-1-kinase* *Dephosphorylation* *Glycolytic enzyme*

1. INTRODUCTION

Ingebritsen and Cohen [3] have classified all protein phosphatases involved in the dephosphorylation of phosphoenzymes of intermediary metabolism. They arrived at the conclusion that these dephosphorylations are catalyzed by only four different protein phosphatases, termed phosphatase 1, 2A, 2B and 2C.

We have recently described the purification and characterization of a protein phosphatase from rat liver, termed 6-phosphofructo-1-kinase phosphatase (PFK-phosphatase) [1], which catalyzes the dephosphorylation of all phosphoenzymes of the glycolytic/gluconeogenetic pathway known so far for rat liver, namely 6-phosphofructo-1-kinase (EC 2.7.1.11), fructose-1,6-bisphosphatase (EC 3.1.3.11), pyruvate kinase (EC 2.7.1.40) and 6-phosphofructo-2-kinase (2.7.1.-).

This protein phosphatase shares several properties with another protein phosphatase from rat liver, first described by Hiraga et al. [2] and classified as phosphatase 2C [3]. It was completely dependent on Mg^{2+} (or Mn^{2+}), was independent of calcium, could not be inhibited by either inhibitor 2 or trifluoperazine, and catalyzed preferentially the dephosphorylation of the α-subunit of phosphorylase kinase. However, PFK-phosphatase exhibited certain properties which seemed to be different from those of protein phosphatase 2C: (1) protein phosphatase 2C has been described as a monomeric enzyme of M_r 45000–55000, whereas PFK-phosphatase seemed to be a dimer with an apparent M_r of about 90000; (2) histone H2B was a good substrate for phosphatase 2C [2,3], but not for PFK-phosphatase [1].

Because of these discrepancies we have reexamined this problem and found that PFK-phosphatase and phosphatase 2C are the same enzyme, but that, in contrast to earlier reports [2,3], protein phosphatase 2C is a dimeric enzyme with a molecular mass of about 90 kDa.

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2. MATERIALS AND METHODS

2.1. *Methods*

Preparation of PFK-phosphatase, the measurement and definition of phosphatase activity, the purification and phosphorylation of phosphatase substrates have been described previously [1]. Protein phosphatase 2C was prepared according to [2]. Marker enzyme activities (catalase from beef liver, alcohol dehydrogenase and hexokinase from yeast, lactate dehydrogenase and aldolase from rabbit muscle) were measured as in [5]. 6-Phosphofructo-2-kinase was prepared and its activity measured as in [6]. All other proteins were localized and quantified by SDS polyacrylamide gel electrophoresis [7]. Two-dimensional gel electrophoresis was performed as in [8]. Histone-Sepharose 4B was prepared and the chromatography performed as in [2].

2.2. *Gel filtration experiments*

For the gel filtration experiments two rat livers were homogenized, centrifuged and the supernatant adsorbed on a DEAE-cellulose column (2 × 12 cm) equilibrated in buffer A – 20 mM triethanolamine HCl (pH 7.5) at 4°C, 2 mM EDTA, 0.2 mM EGTA, 0.5 mM $MgCl_2$ and 1 mM 1,4-dithioerythritol – as in [1]. All further steps were performed in buffer A at 4°C. The column was washed with 80 mM NaCl and the protein phosphatase activities eluted with 200 mM NaCl. The proteins were concentrated by fractionated ammonium sulfate precipitation (176 mg/ml and 390 mg/ml). The sediment of the second precipitation step was dissolved in 3 ml, centrifuged and applied to the gel filtration column (2 × 90 cm, Sephadex G-100 or Sephacryl S-200).

2.3. *Sucrose density gradient centrifugation experiments*

For sucrose gradient centrifugation [4] a SW 50.1 swinging bucket rotor (Beckman Instruments, München) was used. The gradient consisted of 4.6 ml of the 5–20% sucrose gradient solution in buffer A. A total sample volume of 100 μl was layered on top of the gradient, followed by centrifugation at 45000 rpm for 5 h at 4°C. After the run the tubes were punctured and fractions of 160 μl were collected. The following proteins were used: catalase (0.5 mg/ml) and alcohol dehydrogenase (0.2 mg/ml) as standard enzymes, hexokinase (2 mg/ml) and bovine serum albumin (0.5 mg/ml) as control proteins and 50 μl of purified protein phosphatases. The phosphatases were dialysed overnight against buffer A to remove glycerol.

2.4. *Materials*

CNBr-activated Sepharose 4B, Sephadex G-100 and Sephacryl S-200 were obtained from the Deutsche Pharmacia (Freiburg, FRG), DEAE-cellulose DE 52 from Whatman (Springfield, England) and histones from Sigma Chemie (Taufkirchen, FRG). Biochemicals, auxiliary enzymes used for enzyme activity measurements as well as calibration proteins came from Boehringer (Mannheim, FRG). All other chemicals (analytical grade) were purchased from E. Merck (Darmstadt, FRG). [γ-^{32}P]ATP for the preparation of labeled substrates came from Amersham-Buchler (Braunschweig, FRG).

3. RESULTS AND DISCUSSION

3.1. *Gel filtration experiments*

The preparations of phosphatase 2C [2] and PFK-phosphatase [1] both from rat liver are very similar. They differ only in the choice of the material for gel chromatography and in the last purification step. Hiraga et al. [2] performed gel filtration on Sephadex G-100 columns while we used Sephacryl S-200 for this step [1]. The last step in the procedure of Hiraga et al. was chromatography over histone-Sepharose 4B whereas we used FPLC-chromatography over Mono Q and subsequent sucrose gradient centrifugation as final steps. To reexamine the behavior of the phosphatases during gel filtration we prepared a rat liver 100000 × g supernatant according to [1]. After loading this supernatant on a DEAE-cellulose column and washing with 80 mM NaCl, the protein phosphatases were eluted with 200 mM NaCl. This eluate should contain PFK-phosphatase activity as well as phosphatase 2C as this enzyme emerges from DEAE-cellulose at 120 mM [2] or 200 mM NaCl [9], while PFK-phosphatase elutes at 120–150 mM NaCl [1]. The proteins were concentrated by ammonium sulfate precipitation (0.39 g/ml) and chromatographed on a Sephadex G-100 column. The fractions were

tested for PFK-phosphatase as well as for pyruvate kinase phosphatase activity. According to the molecular mass previously reported PFK-phosphatase should elute with about $M_{r(app.)}$ 90000, while phosphatase 2C was expected to elute later with about $M_{r(app.)}$ 45000–55000. The results are depicted in fig.1.

PFK-phosphatase as well as pyruvate kinase phosphatase appeared in two peaks, one emerging with the void volume, the second clearly after bovine serum albumin and shortly before ovalbumin. According to [9] the first peak eluting with the void volume represents phosphatase $2A_1$ and the second the Mg^{2+}-dependent phosphatase 2C. No peak of PFK-phosphatase activity emerged with $M_{r(app.)}$ 90000, although this had to be expected on the basis of former results obtained by chromatography on Sephacryl S-200 [1]. Evidently PFK-phosphatase behaves during gel filtration on Sephadex G 100 in the same way as phosphatase 2C.

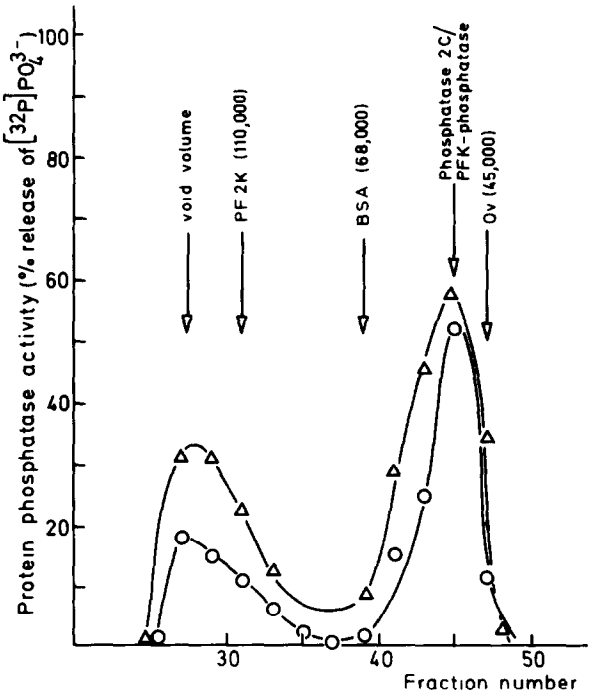

Fig.1. Gel filtration of phosphatase 2C and PFK-phosphatase on Sephadex G-100 was performed as described in section 2. The assays contained 8 mM Mg^{2+} but no Mn^{2+}. According to [3] pyruvate kinase was used as substrate for protein phosphatase 2C. The phosphatase 2C/PFK-phosphatase emerging with approx. $M_{r(app.)}$ 55000 was completely dependent on Mg^{2+} (not shown). (▽—▽) Pyruvate kinase phosphatase, (○—○) 6-phosphofructo-1-kinase phosphatase; PF2K, 6-phosphofructo-2-kinase; BSA, bovine serum albumin; Ov, ovalbumin.

If PFK-phosphatase and phosphatase 2C were identical, protein phosphatase 2C should emerge from a Sephacryl S-200 column with the same apparent molecular mass as PFK-phosphatase. This is indeed the case, as shown in fig.2a. We found only one peak of Mg^{2+}-dependent protein phosphatase activity and that eluted with an apparent M_r of 92000. When this phosphatase activity was subsequently concentrated and chromatographed on Sephadex G-100 (fig.2b), again a single peak of Mg^{2+}-dependent protein phosphatase activity appeared now with $M_{r(app.)}$ 55000. In fig.2a and b (insets) we depicted the calibration curves for the estimation of the apparent molecular masses of the protein phosphatases on Sephacryl S-200 and on Sephadex G-100, respectively. We used 10 different proteins for the calibration. Only two of them fell off the curve for the Sephacryl column, but four proteins did not fit the calibration curve for the Sephadex column. The change in the ratio of activities for PFK-phosphatase and pyruvate kinase phosphatase represents different 6-phosphofructo-1-kinase concentrations in the assays (pyruvate kinase 0.25 μg/ml in fig.2a and b; 6-phosphofructo-1-kinase 0.1 μg/ml in fig.2a and 0.06 μg/ml in fig.2b).

3.2. *Sucrose density gradient centrifugation*

As a decision seemed only possible when the molecular mass was determined by an additional independent method, we used sucrose density gradient centrifugation. According to [4] the sedimentation coefficient or approximate molecular mass of an unknown enzyme may be determined by a simple ratio of mobilities when a well characterized standard enzyme has been added to the protein mixture. As standard enzymes we used catalase and alcohol dehydrogenase. Hexokinase and bovine serum albumin serving as control proteins fit reasonably well with the calibration curve given by the two standard enzymes (inset fig.3). The purified PFK-phosphatase [1] and phosphatase 2C [2] banded at exactly the same position (fig.3). The apparent M_r values of the control proteins bovine serum albumin and hexokinase and the two

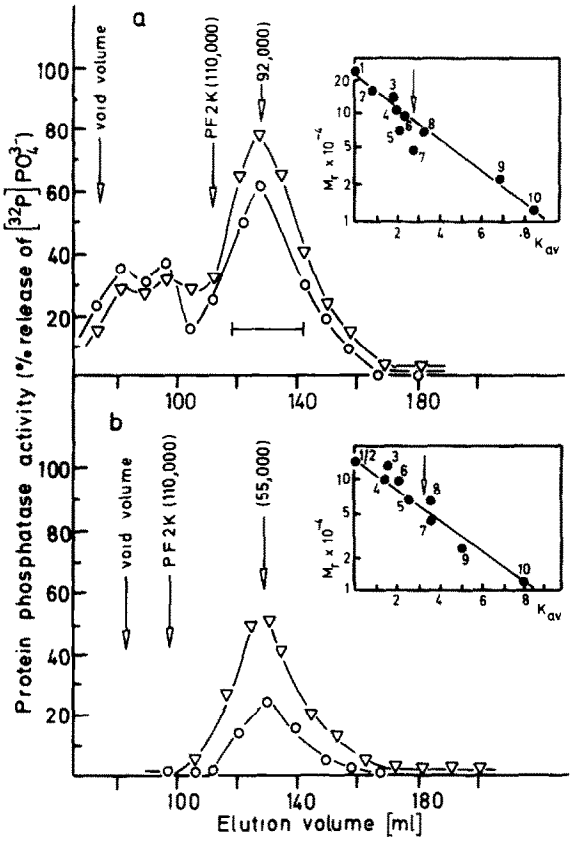

Fig.2. Gel filtration of protein phosphatase 2C and PFK-phosphatase containing fractions of the DEAE-cellulose step on Sephacryl S-200 superfine (a) followed by gel filtration on Sephadex G-100 (b) as described in section 2. The phosphatase activity emerging with an apparent M_r of 90000 from the Sephacryl S-200 column was pooled (horizontal bar), precipitated with 390 mg/ml ammonium sulfate, dissolved in 2 ml buffer A, and applied to the Sephadex G-100 column. For assay conditions, symbols and abbreviations see fig.1. Insets: calibration curves obtained with calibration proteins. 1, void volume (Ferritin); 2, aldolase; 3, lactate dehydrogenase; 4, 6-phosphofructo-2-kinase; 5, bovine serum albumin; 6, hexokinase; 7, ovalbumin; 8, hemoglobin; 9, chymotrypsinogen A; 10, cytochrome *c*; the arrow indicates the elution position of PFK-phosphatase/protein phosphatase 2C; the molecular mass is given in a logarithmic scale.

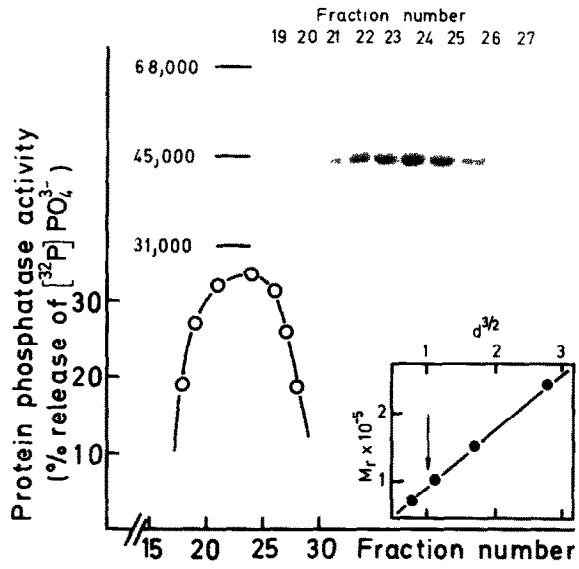

Fig.3. Comparison of the activity of PFK-phosphatase in fractions of a sucrose density gradient with the corresponding protein pattern obtained by SDS polyacrylamide gel electrophoresis. Experimental details are given in section 2. PFK-phosphatase sedimented in the sucrose gradient with about $M_{r(app.)}$ 90000 (see inset). Aliquots (60 μl) of the gradient fractions containing PFK-phosphatase activity were directly used for SDS polyacrylamide electrophoresis, where the protein migrated with $M_{r(app.)}$ 44500. Marker proteins for the SDS electrophoresis: bovine serum albumin (M_r 68000), ovalbumin (M_r 45000), carbonic anhydrase (M_r 31000). Inset: plot according to [4] showing the sedimentation behavior of purified phosphatase 2C and PFK-phosphatase (arrow), standard proteins (1, catalase; 2, alcohol dehydrogenase) and control proteins (3, hexokinase; 4, bovine serum albumin) in linear 5–20% sucrose density gradients. The plot represents one out of 7 centrifugation experiments. The molecular mass is given in a logarithmic scale; d, distance from meniscus.

phosphatases obtained from 7–9 experiments are 71400 [9], 86400 [9] and 87000 [8] with respect to catalase and 75150 [8], 91400 [8] and 91500 [7] with respect to alcohol dehydrogenase as standard enzymes. Accordingly, the M_r values of the protein phosphatase activities lie between 87000 and 91500, which compares reasonably well with the $M_{r(app.)}$ 92000 obtained by Sephacryl S-200 gel filtration.

When the Mg^{2+}-dependent protein phosphatase sedimenting at about M_r 90000 in the sucrose gradient was subsequently analyzed by SDS polyacrylamide gel electrophoresis, it banded with about $M_{r(app.)}$ 44500. A 2-D electrophoresis of the purified enzyme revealed only one spot of M_r

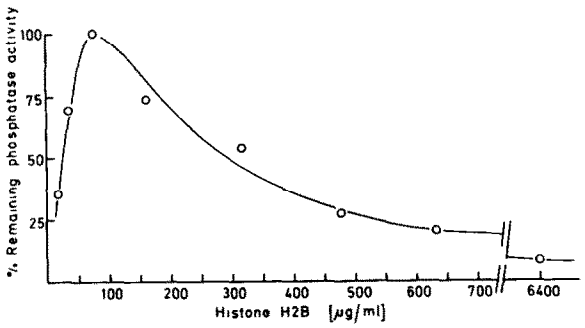

Fig.4. Dependence of PFK-phosphatase activity on histone H2B concentration. Increasing [^{32}P]histone H2B concentrations were incubated for 5 min at 30°C with 0.25 mU of purified PFK-phosphatase in a total volume of 10 μl. The reaction was stopped, the proteins precipitated and counted for radioactivity as described in [1].

44500 (not shown) indicating that the 90-kDa enzyme consists of two identical subunits.

Accordingly, the Mg^{2+}-dependent protein phosphatase described by Mieskes et al. [1] and protein phosphatase 2C purified according to Hiraga et al. [2] have exactly the same molecular mass. As shown here the lower molecular mass of protein phosphatase 2C reported previously [2,3] has resulted from the irregular behavior of the enzyme during Sephadex G-100 chromatography.

We have, in addition, chromatographed both protein phosphatase preparations (PFK-phosphatase and protein phosphatase 2C) on a histone-Sepharose 4B column under conditions given in [2]. Both preparations showed only one Mg^{2+}-dependent protein phosphatase with a maximum at the same ionic strength (not shown).

One discrepancy still remained: we reported for PFK-phosphatase a dephosphorylation ratio for the substrate pair pyruvate kinase/histone H2B of 4 [1], while for phosphatase 2C a ratio of about 0.25 had been reported [3]. The main methodological difference was an about 30 times higher histone concentration (1 mg/ml *vs* 0.03 mg/ml) in the assay of PFK-phosphatase. Fig.4 demonstrates that increasing histone H2B concentrations inhibit PFK-phosphatase activity. When tested at the concentrations of histone H2B used in [3] we found an almost 7-times higher PFK-phosphatase activity than had been reported before [1] and the dephosphorylation ratio for the substrate pair pyruvate kinase/histone H2B became about 0.5–0.6. This is not significantly different from the ratio reported in [3].

On the basis of these results the following conclusions can be drawn: (1) the PFK-phosphatase described in [1] is not a distinct protein phosphatase but identical with protein phosphatase 2C as defined in [3]; (2) the molecular mass of 45–55 kDa for protein phosphatase 2C reported in [2,3] has resulted from the irregular behaviour of the enzyme during Sephadex G-100 gel chromatography. According to the results of two independent methods, native protein phosphatase 2C has an apparent M_r of 90000 and consists of two probably identical subunits. The fact that protein phosphatase 2C is a dimer could be of importance with respect to the regulation of this enzyme.

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