Contribution of different protein phosphatases to the dephosphorylation of 6-phosphofructo-1-kinase and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in rat liver

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The nature of rat liver protein phosphatases involved in the dephosphorylation of the glycolytic key enzyme 6-phosphofructo-1-kinase and the regulatory enzyme 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase was investigated. In terms of the classification system proposed by Ingebritsen & Cohen [(1983) *Eur. J. Biochem.* **132**, 255–261], only the type-2 protein phosphatases 2A (which can be separated into 2A₁ and 2A₂) and 2C act on these substrates. Fractionation of rat liver extracts by anion-exchange chromatography and gel filtration revealed that protein phosphatase 2A is responsible for most of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase phosphatase activity (activity ratio 2A/2C = 4:1). On the other hand, 6-phosphofructo-1-kinase phosphatase activity is equally distributed between protein phosphatases 2A (2A₁ plus 2A₂) and 2C. In addition, the possible role of low-*M_r* compounds for the control of purified protein phosphatase 2C was examined. At near-physiological concentrations, none of the metabolites studied significantly affected the rate of dephosphorylation of 6-phosphofructo-1-kinase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, pyruvate kinase or fructose-1,6-bisphosphatase.

6-Phosphofructo-1-kinase (PF1K) is considered to be the rate-limiting enzyme in the control of glycolysis in muscle and liver. It was therefore of great interest when Van Schaftingen *et al.* (1980) identified fructose 2,6-bisphosphate as the most potent allosteric activator of PF1K. This metabolite is synthesized from and degraded to fructose 6-phosphate by a bifunctional enzyme called 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PF2K/F2,6BPase; Hers & Hue, 1983; Pilkis *et al.*, 1984). PF1K and PF2K/F2,6BPase undergo reversible phosphorylation/dephosphorylation in the intact cell. Phosphorylation of both enzymes is probably catalysed by a cyclic AMP-dependent protein kinase (El-Maghrabi *et al.*, 1982; Brand *et al.*, 1983; Söling, 1984). However, the nature of the protein phosphatases responsible for the dephosphorylation of the two enzymes is not clear.

We have previously described the purification of a PF1K phosphatase which was also able to dephosphorylate PF2K/F2,6BPase (Mieskes *et al.*, 1984), and subsequently identified this enzyme as protein phosphatase 2C (by the classification system of Ingebritsen & Cohen, 1983). However, it is not known which of the four protein phosphatases described by Ingebritsen & Cohen (1983) could be responsible for the dephosphorylation of PF1K and PF2K/F2,6BPase, nor the quantitative contribution made by each one.

In the present study we have measured the relative contributions of each of the four protein phosphatases to the dephosphorylation of PF1K and PF2K/F2,6BPase in rat liver. According to the results obtained, protein phosphatases 2A and 2C each contribute about 50% to the dephosphorylation of PF1K, whereas the dephosphorylation of PF2K/F2,6BPase is catalysed mainly by protein phosphatase 2A (activity ratio 2A/2C = 4:1). While this work was in progress, we were informed of similar work from another laboratory (Pelech *et al.*, 1984). Their results are in general agreement with ours.

Abbreviations used: PF1K, 6-phosphofructo-1-kinase (EC 2.7.1.11); PF2K/F2,6BPase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (EC 2.7.1.-).

Experimental

Methods

Phosphorylase kinase (13650 nmol of phosphate/min per mg at pH 8.2) and phosphorylase *b* from rabbit muscle were kindly given by Dr. H. P. Jennissen (University of Munich, Germany) and Dr. P. Cohen (University of Dundee, U.K.). The catalytic subunit of the cyclic AMP-dependent protein kinase was prepared from bovine liver as described by Peters *et al.* (1977). The heat-stable inhibitor-2 was prepared exactly as described by Yang *et al.* (1981). The preparations of protein phosphatase 2C (PF1K phosphatase), 6-phosphofructo-1-kinase and all further experimental details have been described previously (Mieskes *et al.*, 1984). PF2K/F2,6BPase was prepared as described by El-Maghrabi *et al.* (1982), with the following modifications: all steps except the fast protein liquid chromatography were performed in 20 mM-Tes (2-[2-hydroxy-1,1-bis(hydroxymethyl)amino]-ethanesulphonic acid), pH 7.5, containing 10 mM-KCl, 0.1 mM-EDTA, 1 mM-1,4-dithioerythritol, 0.1 mM-phenylmethanesulphonyl fluoride and leupeptin (0.5 µg/ml). DEAE-Sephadex was replaced by DEAE-cellulose and the enzyme was eluted with a step of 150 mM-KCl in the above buffer; the phosphocellulose chromatography was followed by a final purification on Mono Q (fast protein liquid chromatography) under conditions described for the purification of PF1K phosphatase. For fast protein liquid chromatography the Tes was replaced by 20 mM-Tris and the proteinase inhibitors were omitted.

PF1K was labelled by using the catalytic subunit of the cyclic AMP-dependent protein kinase to a maximum of 1.5–2 mol of phosphate/mol (four subunits), and phosphorylase *b* by using phosphorylase kinase to 0.6–1 mol of phosphate/mol as described by Mieskes *et al.* (1984). PF2K/F2,6BPase was phosphorylated to 1.6–2 mol of phosphate/mol (two subunits) by using the catalytic subunit of the cyclic AMP-dependent protein kinase as described by El-Maghrabi *et al.* (1982). According to the literature (Brand *et al.*, 1983; Ingebritsen & Cohen, 1983; Pilakis *et al.*, 1984), each phosphoprotein should be labelled at one site only.

Phosphoprotein phosphatase activities were measured as described previously (Mieskes *et al.*, 1984). The basic assay mixture did not contain Mg^{2+} or bovine serum albumin. $MgCl_2$ (7.5 mM) and/or $MnCl_2$ (1 mM) were included where specified. The substrate concentrations were 0.2 mg/ml for phosphorylase, 0.1 mg/ml for PF1K and 0.07 mg/ml for PF2K/F2,6BPase. In order to measure phosphatase-catalysed dephosphorylation, the incubation was stopped after 5 or 10 min

and proteins were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The bands corresponding to the labelled substrates were excised and counted for radioactivity.

Separation of protein phosphatases from rat liver cytosol by DEAE-cellulose chromatography

Two rat livers were homogenized, centrifuged (100 000 *g* for 60 min) and the supernatant was adsorbed on a DEAE-cellulose column (2 cm × 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 described by Mieskes *et al.* (1984). All further steps were performed in buffer A at 4°C. The column was washed with 50 mM-NaCl until the absorbance (A_{280}) reached the base-line. Protein phosphatase activities were eluted with a linear gradient of 50–500 mM-NaCl in 300 ml of buffer A with a flow rate of 25 ml/h, and collected in 3.8 ml fractions.

Materials

Sephacryl S-200 and the complete FPLC equipment were obtained from Deutsche Pharmacia (Freiburg, Germany). DEAE-cellulose DE 52 was from Whatman (Maidstone, Kent, U.K.). Biochemicals came from Boehringer (Mannheim, Germany). All other chemicals were purchased from E. Merck (Darmstadt, Germany). [γ - ^{32}P]ATP for the preparation of labelled substrates came from Amersham-Buchler (Braunschweig, Germany).

Results and discussion

In this study we examined only those soluble protein phosphatases from livers of fed rats which could be adsorbed on DEAE-cellulose and subsequently eluted with a gradient of 50–500 mM-NaCl under standardized conditions. Fig. 1 represents the results of such a DEAE-cellulose chromatography with the protein phosphatase pattern measured with labelled phosphorylase *a* as substrate. The assay included 1 mM- Mn^{2+} , because the protein phosphatases 1 and 2A were gradually converted during purification and storage into forms that were partly dependent on Mn^{2+} . By the differentiating criteria given by Ingebritsen & Cohen (1983), the first large double peak eluted under these conditions at about 100–150 mM-NaCl represents protein phosphatase 2A₁ and the active form of phosphatase 1, whereas the second peak represents protein phosphatase 2A₂. Addition of 8 units (for definition of units see Yang *et al.*, 1981) of the heat-stable inhibitor-2 was sufficient to inhibit all protein phosphatase 1, which accounts for about 60% of the first double peak. In contrast, protein phosphatases 2B and 2C have only

Fig. 1. Separation of phosphorylase phosphatases from rat liver cytosol by chromatography on DEAE-cellulose. Preparation of rat liver cytosol, chromatography and activity determinations were performed as given in the Experimental section. The nomenclature of the protein phosphatases follows the classification system of Ingebritsen & Cohen (1983). The assay was performed with labelled phosphorylase *a* in the presence of 1 mM-Mn²⁺. The incubation time was 5 min. The arrows indicate the elution positions of protein phosphatases. ○, Phosphorylase phosphatase; ----, A₂₈₀; —, NaCl concn.

Fig. 2. Separation of protein phosphatases 2A₁ + 2C and 2A₂ from rat liver cytosol by DEAE-cellulose chromatography with PFIK and PF2K/F2,6BPase as substrates.

The protein phosphatases were determined in the presence of 1 mM-Mn²⁺ plus 7.5 mM-Mg²⁺. Further details are given in the Experimental section. The arrows indicate the elution positions of the protein phosphatases. ○, PFIK; △, PF2K/F2,6BPase; ----, A₂₈₀; —, NaCl concn.

negligible phosphatase activities (Ingebritsen *et al.*, 1983; Mieskes *et al.*, 1984).

The protein phosphatase distribution obtained with the substrates PF1K and PF2K/F2,6BPase under similar conditions but in the presence of Mn^{2+} plus Mg^{2+} is depicted in Fig. 2. The peak, eluted at about 100–150 mM-NaCl, represents only protein phosphatases 2A₁ and 2C for the following reasons: neither the addition of the heat-stable inhibitor-2 (8 units) nor the presence or absence of Ca^{2+} or trifluoperazine significantly affected the phosphatase activities measured with PF1K and PF2K/F2,6BPase as substrates (results not shown), indicating that neither protein phosphatase 1 nor 2B has a measurable phosphatase activity with these substrates. The second peak, eluted at 180–

260 mM-NaCl, represents protein phosphatase 2A₂ (cf. Fig. 1). The apparently higher PF1K phosphatase activity as compared with the PF2K/F2,6BPase phosphatase activity in Fig. 2 should not be taken as an indication that PF1K is a better substrate for the two phosphatases, as such a difference can easily result from different specific radioactivities of the [³²P]phosphate in the two substrates, as well as from different substrate concentrations. The important comparison within the context of the present work are the relative phosphatase activities of the different phosphatases with the same substrate.

On the basis of the data presented in Fig. 2, the relative activities of the protein phosphatases 2A₁/2C and 2A₂ with respect to the two enzyme substrates can be calculated. Further discrimination between phosphatases 2A₁ and 2C is only possible by using their different cation-dependences. Phosphatase 2C is absolutely dependent on Mg^{2+} , which can be replaced by Mn^{2+} (Mieskes *et al.*, 1984). In contrast, phosphatase 2A₁ is only partly dependent on Mn^{2+} , which cannot be replaced by Mg^{2+} . Therefore phosphatase activities measured in the presence of Mg^{2+} without Mn^{2+} represent phosphatase 2C plus the remaining activity of the unstimulated phosphatase 2A₁. Phosphatase activities measured without Mg^{2+} and without Mn^{2+} represent only unstimulated

Fig. 3. Determination of protein phosphatase 2C activity by difference measurements with PF1K as substrate after fractionation of rat liver cytosol on DEAE-cellulose. Protein phosphatase 2C (○) was determined as the difference between the phosphatase activities measured in the presence (△) and in the absence (●) of 7.5 mM- Mg^{2+} . All assays were performed without Mn^{2+} . Only the elution range of protein phosphatase 2A₁/2C is shown. —, NaCl concn. Further details are given in the Experimental section.

Fig. 4. Determination of protein phosphatase 2C activity by difference measurements with PF2K/F2,6BPase as substrate after fractionation of rat liver cytosol on DEAE-cellulose.

Symbols and details are the same as in Fig. 3. Activity measurements were carried out in the absence and presence of 7.5 mM- Mg^{2+} , but without Mn^{2+} .

phosphatase 2A₁. The difference between these two measurements gives the activity of protein phosphatase 2C. When this activity is subtracted from that measured in the presence of Mg²⁺ plus Mn²⁺, one obtains the activity of protein phosphatase 2A₁. This procedure is of course only possible as long as no other protein phosphatase activity acting on the same substrate is present: protein phosphatases 1 and 2B can be excluded, as they do not react with PF1K and PF2K/F2,6BPase, as mentioned above. By the above criteria, there was no indication for the interference by additional protein phosphatase activities not contained in the scheme of Ingebritsen & Cohen (1983).

In Figs. 3 and 4 the activities of protein phosphatases 2A₁ and 2C with the substrates PF1K and PF2K/F2,6BPase respectively are depicted. The activities of protein phosphatase 2A₂ with the two substrates can be calculated from measurements performed in the presence of Mn²⁺ plus Mg²⁺ (as for instance depicted in Fig. 2). These data allow calculation of the relative contributions of protein phosphatases 2A₁, 2A₂ and 2C to the total dephosphorylation activity with either PF1K or PF2K/F2,6BPase as substrate: for PF1K as substrate about 50% of the total PF1K phosphatase activity can be attributed to phosphatase 2C, 17% to phosphatase 2A₁ and 33% to phosphatase 2A₂. For PF2K/F2,6BPase as substrate the distribution of phosphatase activity is clearly different in that 20% is contributed by phosphatase 2C, 30% by phosphatase 2A₁ and 50% by phosphatase 2A₂.

Separation of the two phosphatases 2A₁ and 2C by gel filtration on Sephacryl S-200 (Fig. 5) gives an independent measurement of the relative activities of these two protein phosphatases with the two enzyme substrates. From the results obtained, the activity ratio for phosphatase 2C/2A₁ is 3:1 for PF1K and 1:1.7 for PF2K/F2,6BPase. This is in good agreement with the results obtained from difference measurements of the type depicted in Figs. 2–4, which gave 3:1 and 1:1.6 with PF1K and PF2K/F2,6BPase as substrates respectively.

Protein phosphatase 2A₂ (Fig. 1) most likely results from the conversion of phosphatase 2A₁ into 2A₂ by the loss of a 58 kDa component during purification (Ingebritsen *et al.*, 1983). Therefore it seems reasonable to compare the total activity of protein phosphatase 2A with that of protein phosphatase 2C. Such a comparison reveals activity ratios of phosphatase 2A_{total}-phosphatase 2C of 1:1 for PF1K and of 4:1 for PF2K/F2,6BPase. Accordingly protein phosphatase 2A is the major PF2K/F2,6BPase phosphatase, whereas PF1K phosphatase activity is distributed almost equally between protein phosphatases 2A and 2C.

Fig. 5. Separation of protein phosphatases 2A₁ and 2C by gel filtration on Sephacryl S-200

After fractionation on DEAE-cellulose, the protein phosphatase activities eluted in the range 90–100 mM-NaCl were combined, concentrated by (NH₄)₂SO₄ precipitation (390 mg/ml) and applied to a Sephacryl S-200 column (2 cm × 90 cm) equilibrated in buffer A. The column was developed at a flow rate of 15 ml/h. Fractions (3.8 ml) were collected and assayed for phosphorylase phosphatase activity (—) in the presence of 1 mM-Mn²⁺, or for PF1K-phosphatase activity (○) and PF2K/F2,6BPase-phosphatase activity (△), both in the presence of 1 mM-Mn²⁺ plus 7.5 mM-Mg²⁺. Control incubations for PK1K phosphatase without Mn²⁺ and Mg²⁺ (●) demonstrate the complete dependence of protein phosphatase 2C on bivalent cations. The column was calibrated as described previously (Mieskes *et al.*, 1984).

This relationship could theoretically be modified in intact cells by physiological effectors. Therefore the influence of various metabolites on the dephosphorylation of PF1K, PF2K/F2,6BPase, pyruvate kinase and fructose-1,6-bisphosphatase with purified protein phosphatase 2C was examined (Table 1). In no case was an activation of the dephosphorylation reaction observed, and significant inhibition occurred only at unphysiologically high concentrations. Inhibition by high concentrations of effectors was in most cases not selective for a specific phospho-enzyme substrate, indicating that the effectors acted mainly on the protein phosphatase rather than on the substrates. However, as the effectors failed to be effective at physiological concentrations, one has to assume that, for protein

Table 1. *Effect of various metabolites on the rate of dephosphorylation of glycolytic/gluconeogenic enzymes by purified protein phosphatase 2C*

Activities are expressed as percentage of those of control incubations from which the metabolites were omitted. For each value the time-dependence of the dephosphorylation reaction was measured. The initial velocity given by the first 2 min of this curve represents the protein phosphatase activities. Abbreviations: PK, L-type pyruvate kinase (EC 2.7.1.40); F1,6BPase fructose-1,6-bisphosphatase (EC 3.1.3.11); both enzymes were prepared and labelled as described by Mieskes *et al.* (1984).

Metabolite	Metabolite concn. (mM)	Phosphoenzyme used as substrate			
		PF1K	PF2K/ F2,6BPase	PK	F1,6BPase
Citrate	1	71	71		
6-Phosphogluconate	0.1	95	86		73
	1	36		58	60
Glucose 6-phosphate	1	94	71	80	
Fructose 6-phosphate	1	94	71	100	
Fructose 1,6-bisphosphate	0.1	74	69		91
	1	44		70	33
α -Glycerophosphate	1	48	100	75	
2-Phosphoglycerate	0.1	70	100		
	1	36		51	49
3-Phosphoglycerate	0.1	93	100	65	100
	1	42			60
2,3-Bisphosphoglycerate	1	55			
	5	15			
Phosphoenolpyruvate	0.5	67			
	1	50	68	68	64
	5	0			
Pyruvate	1			100	
Lactate	1			100	
Alanine	1	85		100	80
Glucose	1	91			
Fructose 2,6-bisphosphate	0.01	69		82	
	0.02		85		
	0.1	57		100	95
	1	33		0	51
AMP	0.1		71		
	1	88			
ADP	0.2	89			
MgATP	1	94	46		

phosphatase 2C, the rate of dephosphorylation of the phospho-enzymes studied is mainly regulated by the concentration of the phospho-enzyme substrate itself.

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