Effects of phosphorylation on the kinetic properties of rat liver fructose-1,6-bisphosphatase

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A new purification procedure for rat liver fructose-1,6-bisphosphatase that involves use of Procion Red–Sephacrose is described. The purified enzyme was homogeneous, had a subunit M_r of 40 000–41 000 and seemed to be undegraded. The enzyme could be phosphorylated by cyclic AMP-dependent protein kinase with a stoichiometry of one per subunit. Phosphorylation caused a 2-fold decrease in the K_m of the enzyme for fructose 1,6-bisphosphate, but did not affect its allosteric responses to AMP, Mg^{2+} and fructose 2,6-bisphosphate.

Fructose-1,6-bisphosphatase (D-fructose 1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) is thought to play an important role in the control of carbohydrate metabolism in mammalian liver (see, e.g., Hers & Hue, 1983; Horecker *et al.*, 1975). The regulation of the enzyme has been the subject of intensive study; the enzyme is inhibited sigmoidally by AMP (Taketa & Pogell, 1965) and by fructose 2,6-bisphosphate (Pilkis *et al.*, 1981; Van Schaftingen & Hers, 1981b), and it is activated sigmoidally by bivalent metal cations (Nimmo & Tipton, 1975b). Rat liver fructose-1,6-bisphosphatase can be phosphorylated by cyclic AMP-dependent protein kinase (Hosey & Marcus, 1981; Riou *et al.*, 1977). However, it is now clear that the fructose-1,6-bisphosphatases from mouse liver, rabbit liver and pig kidney cannot be so phosphorylated; in comparison with the enzyme from these sources, the rat liver enzyme contains a C-terminal extension in which the phosphorylation site is located (Hosey & Marcus, 1981; Rittenhouse *et al.*, 1983).

There has been some disagreement over the effects of phosphorylation on the kinetic properties of rat liver fructose-1,6-bisphosphatase. Riou *et al.* (1977) showed that phosphorylation caused a 40% increase in V with no change in the K_m for fructose 1,6-bisphosphate and the apparent K_i for AMP. Ekman & Dahlqvist-Edberg (1981) observed not only a similar small increase in V but also a 2-fold decrease in the K_m for fructose 1,6-bisphosphate. On the other hand, Rittenhouse *et al.* (1983)

reported that phosphorylation caused no detectable changes in several kinetic properties of the enzyme. However, none of these groups studied the effects of phosphorylation of the enzyme on the interactions between AMP, fructose 2,6-bisphosphate and bivalent metal cations. We therefore decided to investigate systematically the kinetic properties of the phosphorylated and non-phosphorylated forms of rat liver fructose-1,6-bisphosphatase.

Since mammalian liver fructose-1,6-bisphosphatases can be modified proteolytically during purification, and this can affect their regulatory properties (see, e.g., Horecker *et al.*, 1975), we devised a new purification procedure for the rat liver enzyme that gives 'native' undegraded enzyme. We show in the present paper that phosphorylation causes a significant decrease in the K_m of the enzyme for fructose 1,6-bisphosphate, but does not alter its other kinetic properties. In the accompanying paper (Meek & Nimmo, 1984) we present a new model for the allosteric properties of rat liver fructose-1,6-bisphosphatase.

Experimental

Enzymes

Rabbit muscle phosphorylase kinase, phosphorylase *b* and phosphorylase *a* and ox liver fructose-1,6-bisphosphatase were prepared as described previously (Nimmo & Tipton, 1975a; Nimmo *et al.*, 1976). The catalytic subunit of cyclic AMP-dependent protein kinase was prepared from ox liver by the method of Sugden *et al.* (1976), except that the final gel-filtration step was omitted. Other

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enzymes were obtained from Boehringer Corp. (London), Lewes, Sussex, U.K.

Chemicals

Fructose 1,6-bisphosphate, triethanolamine hydrochloride and all nucleotides were obtained from Boehringer. Benzamidine hydrochloride, bovine serum albumin, histone type IIA and phenylmethanesulphonyl fluoride were from Sigma (London) Chemical Co., Poole, Dorset, U.K. DEAE-cellulose (DE52) was obtained from Uniscience, Cambridge, U.K. Sepharose and Sephadex gels were obtained from Pharmacia (G.B.), Milton Keynes MK9 3HP, U.K. Radiochemicals were obtained from Amersham International, Amersham, Bucks., U.K. Fructose 2,6-bisphosphate was prepared by a minor modification (Meek & Nimmo, 1983) of the method of Van Schaftingen & Hers (1981a). Procion Red HE3B was given by Dr. L. Jervis, Department of Biology, Paisley College of Technology. All other chemicals were of A.R. grade from BDH Chemicals, Poole, Dorset, U.K. Sepharose 6B was cross-linked (Easterday & Easterday, 1974) and Procion Red-Sepharose was then synthesized as described by Borthwick *et al.* (1984).

Enzyme assays

Fructose-1,6-bisphosphatase was assayed at 37°C by coupling the production of fructose 6-phosphate to the reduction of NADP⁺. One unit of enzyme activity is defined as the amount needed to catalyse the formation of 1 μ mol of product/min. The standard assay conditions at pH 7.2 and pH 9.5 were those given by Nimmo & Tipton (1975a). The 'pH ratio' of the enzyme is defined as its activity at pH 7.2 divided by its activity at pH 9.5. The sensitivity of the enzyme to inhibition by 50 μ M-AMP was measured at pH 7.2. For kinetic experiments the fluorimetric procedure described by Meek & Nimmo (1983) was used. Each experimental point is the mean of triplicate assays. Double-reciprocal plots were analysed as described by Meek & Nimmo (1983). The concentrations of Mg²⁺, AMP, fructose 1,6-bisphosphate and fructose 2,6-bisphosphate refer to the free species and were calculated by using the association constants given by Meek & Nimmo (1983).

Cyclic AMP-dependent protein kinase was assayed at 30°C by using the phosphocellulose-paper method of Witt & Roskoski (1975). Incubations contained 20 mM-potassium phosphate, 2 mM-MgCl₂, 0.2 mM-[γ -³²P]ATP (10⁴ c.p.m./nmol), 0.01 mM-cyclic AMP and histone (2 mg/ml). One unit of protein kinase activity is defined as the amount needed to catalyse the incorporation of 1 pmol of phosphate into histone/min. Marker

enzymes used in gel-filtration experiments were assayed by routine spectrophotometric methods.

Purification of rat liver fructose-1,6-bisphosphatase

All steps were performed as rapidly as possible at 0–4°C. Livers from six Wistar rats, fed on a normal diet, were homogenized in 4 vol. of 0.25 M-sucrose containing 2 mM-MgCl₂, 1 mM-EDTA, 0.4 mM-dithiothreitol and 1 mM-benzamidine hydrochloride. A solution of phenylmethanesulphonyl fluoride (6 mg/ml in ethanol) was added immediately to give a final concentration of 1.2 mM. A particle-free supernatant was prepared by centrifugation at 23000g for 30 min (step 1). The proteins that precipitated between 30% and 50% saturation with (NH₄)₂SO₄ (Dixon, 1953) were collected; the pH was maintained in the range 7.0–7.4 during this operation. The precipitate was dissolved in 20 mM-triethanolamine hydrochloride/KOH, pH 7.5, containing 1 mM-MgCl₂, 0.4 mM-dithiothreitol and 1 mM-benzamidine hydrochloride (buffer A) and equilibrated in this buffer by gel filtration on Sephadex G-25 (step 2).

The enzyme was then loaded on a column (4.4 cm $\times$ 8 cm) of DEAE-cellulose equilibrated with buffer A. The column was washed with this buffer and then developed with a 1200 ml linear gradient of 0–0.2 M-KCl in buffer A. Enzyme activity was eluted as a single peak in the range 0.07–0.12 M-KCl. Peak fractions were pooled and dialysed overnight against buffer A (step 3). The material was then loaded on a column (4.4 cm $\times$ 4 cm) of Procion Red-Sepharose equilibrated in buffer A. The column was washed with this buffer and then with buffer A containing 0.1 mM-AMP and 0.1 mM-fructose 1,6-bisphosphate at a flow rate of 180 ml/h. Fructose-1,6-bisphosphatase was specifically eluted as a broad peak with the second wash. The enzyme was concentrated by using a column of DEAE-cellulose (bed volume 1 ml) equilibrated in buffer A; it was eluted from this column with 5 ml of buffer A containing 0.2 M-KCl (step 4).

The concentrated material was loaded on a column (2.2 cm $\times$ 83 cm) of Sephadex G-200 (superfine grade) equilibrated in 20 mM-triethanolamine hydrochloride/KOH, pH 7.5, containing 2 mM-MgCl₂, 1 mM-EDTA, 0.4 mM-dithiothreitol and 1 mM-benzamidine hydrochloride (buffer B). The column was developed with this buffer at a flow rate of 10 ml/h. Enzyme activity was eluted as a single symmetrical peak; the specific activity, 'pH ratio' and AMP-sensitivity of the enzyme were constant across the peak. The enzyme was concentrated by vacuum dialysis, dialysed against buffer B containing 40% (v/v) glycerol and stored at –20°C. The activity, 'pH ratio' and AMP-

sensitivity of the enzyme remained constant for at least 3 months.

Phosphorylation of rat liver fructose-1,6-bisphosphatase

Analytical experiments were performed at 30°C with a mixture (0.2 ml) comprising 35 mM-potassium phosphate, pH 6.8, 0.02 mM-fructose-1,6-bisphosphatase (in terms of subunits), 50 000 units of the catalytic subunit of cyclic AMP-dependent protein kinase/ml, 2.1 mM-MgCl₂, 0.1 mM-EDTA, 0.4 mM-dithiothreitol and 0.2 mM-[γ -³²P]ATP (10⁴ c.p.m./nmol). Protein-bound ³²P was measured in 0.02 ml samples as described by Walsh *et al.* (1971).

To prepare phosphorylated fructose-1,6-bisphosphatase for kinetic analysis this procedure was scaled up 10-fold. When the incorporation of ³²P into protein reached completion, EDTA and NaF were added to final concentrations of 2.5 mM and 100 mM. Phosphorylated fructose-1,6-bisphosphatase was separated from the catalytic subunits and excess ATP by gel filtration on a column (1.1 cm × 32 cm) of Sephadex G-200 equilibrated in 50 mM-triethanolamine hydrochloride / KOH, pH 7.5, containing 100 mM-KCl, 2 mM-EDTA, 10 mM-NaF and 0.1 mM-dithiothreitol. The enzyme was concentrated by vacuum dialysis, dialysed against the above buffer containing 40% (v/v) glycerol and stored at -20°C. The activity, 'pH ratio', AMP-sensitivity and ³²P content of the phosphorylated enzyme remained constant for at least 2 months.

Other methods

Denaturing gel electrophoresis was performed and the marker proteins used were as described previously (Borthwick *et al.*, 1984). Non-denaturing gel electrophoresis was done as described by Nimmo & Tipton (1975a). Sedimentation-equilibrium centrifugation and analytical gel filtration were performed as described by Borthwick *et al.* (1984). Alkali-labile phosphate was determined by the method of Nimmo *et al.* (1976), with glycogen phosphorylases *b* and *a* as control and standard respectively. The amino acid composition of rat

liver fructose-1,6-bisphosphatase was determined by standard methods, and the partial specific volume of the enzyme was calculated to be 0.734 ml/g (Meek, 1982). Protein concentrations were determined by the method of Bradford (1976).

Results and discussion

Purification of rat liver fructose-1,6-bisphosphatase

We have developed a new purification procedure that gives homogeneous undegraded enzyme reproducibly in good yield; the results of this procedure are summarized in Table 1. The specific activity of the purified enzyme, 60 units/mg at 37°C, is comparable with the values obtained by some other groups (Tejwani *et al.*, 1976; Riou *et al.*, 1977; Marcus *et al.*, 1982) and considerably higher than that of the preparation used by Ekman & Dahlqvist-Edberg (1981).

The purified enzyme was homogeneous by the criteria of denaturing and non-denaturing gel electrophoresis. It gave a single band in the denaturing gel systems of Weber & Osborn (1975) and Laemmli (1970). The subunit *M_r* of the enzyme was estimated in each of these systems by comparison of its mobility with those of marker proteins; the values obtained were 40 000 ± 1000 and 41 000 ± 1000 respectively (mean and range of four determinations; results not shown). The native *M_r* of the enzyme was estimated by sedimentation equilibrium centrifugation and gel filtration to be 153 000 ± 5000; there was no evidence of any association/dissociation behaviour. These results confirm that rat liver fructose-1,6-bisphosphatase is a tetramer.

Proteolysis of mammalian liver fructose-1,6-bisphosphatases at the *N*-terminal end results in changes in the pH optimum and AMP-sensitivity of the enzyme (see, e.g., Horecker *et al.*, 1975). We therefore monitored the 'pH ratio' and the AMP-sensitivity of the enzyme throughout the purification. The data shown in Table 1 indicate that, as judged by these criteria, proteolysis did not occur during the purification and that the purified enzyme can be designated as 'native' fructose-1,6-

Table 1. *Purification of rat liver fructose-1,6-bisphosphatase*

The procedure was as described in the Experimental section. At each step the enzyme was assayed at pH 7.2 in the presence and absence of 50 μM-AMP and at pH 9.5. Values of total activity quoted are those at pH 7.2.

Step	Activity (units)	Specific activity (units/mg)	Inhibition by 50 μM-AMP (%)	'pH ratio'	Purification (fold)	Yield (%)
1	1064	0.21	53	4.1	1	100
2	851	0.61	50	3.9	2.9	80
3	649	2.08	50	4.0	9.9	61
4	265	26.5	52	3.9	126	25
5	180	60.0	49	3.9	286	17

bisphosphatase. This conclusion is supported by the results of denaturing gel electrophoresis. In agreement with the work of Marcus's group (Hosey & Marcus, 1981; Rittenhouse *et al.*, 1983), our value for the subunit M_r of rat liver fructose-1,6-bisphosphatase is rather larger than the values reported for the enzyme from other mammalian tissues. Moreover, it has been reported that some procedures for the isolation of the rat liver enzyme give preparations that show several closely spaced bands on denaturing gel electrophoresis (Hosey & Marcus, 1981; Marcus *et al.*, 1982); this may reflect limited proteolysis. In contrast, our purified enzyme appears to be undegraded.

One of the precautions that we took to prevent proteolysis involved use of the serine proteinase inhibitor phenylmethanesulphonyl fluoride. It is possible that this could react with fructose-1,6-bisphosphatase itself and alter the ability of the enzyme to be phosphorylated. We therefore performed one purification with phenylmethane[^{35}S]-sulphonyl fluoride. The purified enzyme contained no detectable ^{35}S radioactivity, showing that it had not reacted irreversibly with the inhibitor.

Phosphorylation of rat liver fructose-1,6-bisphosphatase

The alkali-labile phosphate content of purified rat liver fructose-1,6-bisphosphatase was 0.54 molecule/subunit; this is considerably higher than the value reported by Pilkis *et al.* (1980). This phosphate is presumably not located at the site phosphorylated by cyclic AMP-dependent protein kinase; in agreement with the results of other workers (Riou *et al.*, 1977; Hosey & Marcus, 1981), we found that our purified enzyme could be phosphorylated by this kinase with a stoichiometry of one per subunit (Fig. 1). In contrast, the 'native' fructose-1,6-bisphosphatase from ox liver could not be phosphorylated under identical conditions (results not shown). This enzyme has a subunit M_r of 35 500 (Nimmo & Tipton, 1975a), and so this observation supports the conclusion of Hosey & Marcus (1981) that only the rat liver enzyme, which contains a C-terminal extension relative to other fructose-1,6-bisphosphatases (Rittenhouse *et al.*, 1983), can be phosphorylated.

Pilkis's group have shown that neither AMP nor low concentrations of fructose 1,6-bisphosphate affect the rate of phosphorylation of rat liver fructose-1,6-bisphosphatase (Riou *et al.*, 1977; Pilkis *et al.*, 1980), and we have confirmed these observations (results not shown). However, we found that fructose 2,6-bisphosphate and high concentrations of fructose 1,6-bisphosphate both caused a significant decrease in the rate of phosphorylation (Fig. 1); the presence in addition of AMP (0.2 mM) had no further effect (results not

Fig. 1. Phosphorylation of rat liver fructose-1,6-bisphosphatase

Incubations were carried out as described in the Experimental section. ○, Fructose-1,6-bisphosphatase with no additions; ▲, plus 0.1 mM-fructose 2,6-bisphosphate; △, plus 10 mM-fructose 1,6-bisphosphate. No ^{32}P was incorporated into protein in the absence of either fructose-1,6-bisphosphatase or catalytic subunits (□).

shown). None of these effectors altered the activity of the protein kinase against histone. We have suggested that the enzyme contains an allosteric site for fructose 2,6-bisphosphate to which high concentrations of fructose 1,6-bisphosphate can also bind (Meek & Nimmo, 1983); it seems likely that the decrease in the rate of phosphorylation is caused by binding of the effectors to this site.

Effect of phosphorylation on the activity of rat liver fructose-1,6-bisphosphatase

The phosphorylation of rat liver fructose-1,6-bisphosphatase (Fig. 1) had no effect on its activity when the enzyme was assayed in the standard assay conditions at pH 7.2 (results not shown); in these conditions the enzyme is essentially fully saturated with its substrates. We studied the kinetic properties of the phosphorylated and non-phosphorylated forms of the enzyme carefully. As shown in Fig. 2, both forms gave linear double-reciprocal plots of velocity against fructose 1,6-bisphosphate concentration at 2 mM free Mg^{2+} . The phosphorylated form had a significantly lower K_m for fructose 1,6-bisphosphate ($1.94 \pm 0.06 \mu\text{M}$) than did the non-phosphorylated form ($2.87 \pm 0.16 \mu\text{M}$). In this experiment, slightly different amounts of the two forms were used; there was no significant difference between the values of V for the two forms. This experiment has been performed

Fig. 2. Effect of phosphorylation on the K_m of rat liver fructose-1,6-bisphosphatase for fructose 1,6-bisphosphate. For details, see the Experimental section. □, Non-phosphorylated enzyme; ○, phosphorylated enzyme.

several times with different batches of enzyme; on each occasion phosphorylation decreased the K_m for fructose 1,6-bisphosphate by a factor of 1.5–2. A change in the K_m for fructose 1,6-bisphosphate was observed at all concentrations of free Mg^{2+} tested (0.17–2.00 mM) (results not shown).

The phosphorylated and non-phosphorylated forms of the enzyme had identical pH optima, measured at saturating concentrations of fructose 1,6-bisphosphate, and identical M_r , as judged by gel filtration. We investigated the allosteric responses of the two forms of the enzyme to AMP, Mg^{2+} and fructose 2,6-bisphosphate. We found no significant differences between the phosphorylated and non-phosphorylated forms (results not shown). The data for the non-phosphorylated form are given in the accompanying paper (Meek & Nimmo, 1984) together with a new model for the allosteric behaviour of rat liver fructose-1,6-bisphosphatase.

Our observation that phosphorylation causes a decrease in the K_m of rat liver fructose-1,6-bisphosphatase for fructose 1,6-bisphosphate confirms the report by Ekman & Dahlqvist-Edberg (1981). In contrast, Riou *et al.* (1977) observed only a small increase in V , and Rittenhouse *et al.* (1983) reported that phosphorylation did not change several kinetic properties of the enzyme, including

its K_m for fructose 1,6-bisphosphate. In our hands, phosphorylation did not affect the responses of the enzyme to AMP, Mg^{2+} and fructose 2,6-bisphosphate. In contrast, Ekdahl & Ekman (1984) reported that phosphorylated enzyme was slightly more sensitive than unphosphorylated enzyme to inhibition by AMP and fructose 2,6-bisphosphate. The reasons for these discrepancies are not clear. One could be that the different groups have used rather different assay conditions; Ekman & Dahlqvist-Edberg (1981) showed that the K_m values of the two forms of the enzyme for fructose 1,6-bisphosphate depend critically on the composition of the assay medium. Another could be in differences between the enzyme preparations used by the different groups. Marcus *et al.* (1982) commented that the purification procedure of Riou *et al.* (1977), which was also used by Ekman & Dahlqvist-Edberg (1981), seems to give rise to partially degraded enzyme. We note in the accompanying paper (Meek & Nimmo, 1984) that there seems to be a qualitative difference in the kinetic nature of the response to AMP between several different preparations of rat liver fructose-1,6-bisphosphatase.

Claus *et al.* (1981) reported that the addition of glucagon to rat hepatocytes increased the degree of phosphorylation of fructose-1,6-bisphosphatase; Riou *et al.* (1977), however, observed no such increase in intact rats. The current view is that phosphorylation of fructose-1,6-bisphosphatase, which seems to occur only with the rat enzyme, is not important in the control of liver carbohydrate metabolism (see, e.g., Claus *et al.*, 1983; Hers & Hue, 1983). We do not dissent from this view, even though our results show that phosphorylation of the rat liver enzyme does alter one of its kinetic properties; even in the rat, changes in the concentration of fructose 2,6-bisphosphate are probably of much greater significance for the regulation of fructose-1,6-bisphosphatase than is its phosphorylation.

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