6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase in rat brain

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The concentration of fructose 2,6-bisphosphate in the brain remained stable during starvation and early stages of ischaemia, but decreased in diabetes or after lengthened ischaemia. 6-Phosphofructo-1-kinase activity was also decreased in diabetic and ischaemic animals, whereas 6-phosphofructo-2-kinase was not modified. The concentration of the bisphosphorylated metabolite seems to be remarkably constant under a wide variety of experimental conditions, suggesting that it plays an essential role in the basal activation of 6-phosphofructo-1-kinase. Purified 6-phosphofructo-2-kinase also showed fructose-2,6-bisphosphatase activity with an activity ratio similar to that of the purified heart isoenzyme. The brain enzyme also has a net charge similar to that of the heart isoenzyme. Its activity is not modified by *sn*-glycerol 3-phosphate, and it is more sensitive to citrate than the liver or muscle isoenzyme. Moreover, the enzyme from brain, similarly to that from heart and muscle, is not modified by the cyclic AMP-dependent protein kinase or protein kinase C. A near-full-length cDNA probe from liver hybridized with RNA from brain and heart. In both cases, a major band of 6.8 kb of RNA and a minor one of 4 kb of RNA were detected. All these properties support the hypothesis that brain contains a different isoenzymic form from that of liver and muscle, and it is probably related to the heart isoform.

INTRODUCTION

Fru-2,6- P_2 is a powerful activator of PFK-1 and an inhibitor of fructose-1,6-bisphosphatase, and it is present in all mammalian tissues. It is involved in the glucose-induced stimulation of glycolysis, not only in liver but also in other tissues. Its synthesis and breakdown are catalysed by PFK-2 and FBPase-2 respectively. These two activities belong to separate domains of each subunit of the same homodimeric protein. The liver bifunctional enzyme integrates a number of metabolic and hormonal signals. It is phosphorylated by the cyclic AMP-dependent protein kinase, modulated by different effectors and also regulated by its control expression (Van Schaftingen, 1987; Hue & Bartrons, 1985; Hue & Rider, 1987; Pilkis & El-Maghrabi, 1988).

Biochemical and immunological data also suggest the existence of distinct PFK-2/FBPase-2 isoenzymes: the liver L type, the muscle M type and the heart H type (Hue & Rider, 1987). The L and M types have a common sequence of 438 amino acids which differ only at the N-terminus. In the L isoenzyme, the divergent sequence is 32 residues long and includes the serine phosphorylated by cyclic AMP-dependent protein kinase (Lively *et al.*, 1988; Taniyama *et al.*, 1988; Darville *et al.*, 1989). The bovine heart H type has a sequence of 530 amino acids and, in contrast with liver and muscle isoenzymes, it is phosphorylated by protein kinase C and also by cyclic AMP-dependent protein kinase. The phosphorylation of the enzyme seems not to alter its activity (Rider & Hue, 1986) or at least produces only slight kinetic changes (Kitamura *et al.*, 1988). Distinct mRNAs coding for PFK-2/FBPase-2 have been described: a 2.1–2.2 kb mRNA from rat liver, a 1.9 kb mRNA from rat skeletal muscle and a 6.8 kb mRNA from rat heart have been found (Colosia *et al.*, 1988; Crepin *et al.*, 1989). Furthermore, a 4 kb mRNA from bovine heart has been reported (Sakata & Uyeda, 1990).

Fru-2,6- P_2 is present in various types of cultivated brain cells

at a concentration similar to that in hepatocytes (Pauwels & Trouet, 1984), being also a positive allosteric effector of brain PFK-1 (Foe & Kemp, 1985; Vora *et al.*, 1985; Dunaway *et al.*, 1988). The bisphosphorylated metabolite seems to increase transiently in parallel with Fru-1,6- P_2 and lactate formation in the early stages of ischaemia (Yamamoto *et al.*, 1990), although Ogushi *et al.* (1990) have not found changes after stimulation of brain glycolysis.

The aim of the present work is to study the metabolism of Fru-2,6- P_2 in different metabolic states and to characterize the bifunctional enzyme PFK-2/FBPase-2, in order to illuminate the role of this metabolite in the control of brain glycolysis.

MATERIALS AND METHODS

Chemicals

[γ - 32 P]ATP (3000 Ci/mmol), [α - 32 P]dCTP (3000 Ci/mmol) and nylon membranes were from Amersham. The Mono Q column was from Pharmacia. Streptozotocin was purchased from Sigma. Enzymes and other biochemical reagents were from either Boehringer or Sigma. cDNA^{2k1400} (Colosia *et al.*, 1988), which was used as a probe, was kindly provided by Dr. A. Tauler. Purified protein kinase C from rat brain was kindly given by Dr. E. Rozengurt (Imperial Cancer Research Fund, London, U.K.).

Treatment of animals

Male Sprague–Dawley rats (200–250 g) were used. Diabetes was induced by intravenous injection of streptozotocin (60 mg/kg) dissolved in 50 mM-sodium citrate (pH 4.5) plus 150 mM-NaCl and allowed to develop for 2 weeks. Animals were killed by decapitation and the heads frozen in liquid N₂, or by microwave radiation (Toshiba TMW-6402A), i.e. they received a 4.5 kW dose for 0.8 s either immediately or after a measured time interval in ischaemic animals. Afterwards, brains were removed

Abbreviations used: Fru-6- P , fructose 6-phosphate; Glu-6- P , glucose 6-phosphate; Glu-1,6- P_2 , glucose 1,6-bisphosphate; Fru-1,6- P_2 , fructose 1,6-bisphosphate; Fru-2,6- P_2 , fructose 2,6-bisphosphate; PFK-1, 6-phosphofructo-1-kinase (EC 2.7.1.11); PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105); FBPase-2, fructose-2,6-bisphosphatase (EC 3.1.3.46); DTT, dithiothreitol; PEG, poly(ethylene glycol).

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and frozen in solid CO₂. Blood samples were taken and immediately deproteinized with ZnSO₄ and Ba(OH)₂ as described by Somogyi (1952).

Assay of metabolites

The concentrations of plasma glucose (Kunst *et al.*, 1984) and β -hydroxybutyrate (Williamson & Mellanby, 1974) were measured enzymically. Fru-2,6-P₂ was extracted and determined as described by Van Schaftingen *et al.* (1982). The content of Glu-1,6-P₂ was measured spectrophotometrically (Passonneau *et al.*, 1969). Citrate was assayed fluorimetrically (Möllering, 1984).

Enzyme assays

PFK-1 was extracted from frozen brains in 10 vol. of ice-cold 250 mM-mannitol/10 mM-Hepes/2.5 mM-MgCl₂/1 mM-KF/0.5 mM-EDTA/1 mM-DTT, at pH 7.4, and centrifuged at 40000 *g* for 10 min at 4 °C. PFK-1 was measured in the cytosolic fraction at pH 8.0, as described by Staal *et al.* (1987).

PFK-2 activity was determined by a modification of the method described by Bartrons *et al.* (1983). Brain samples were homogenized in 10 vol. of ice-cold 20 mM-P_i/100 mM-KF/10 mM-EDTA/1 mM-DTT/3 % poly(ethylene glycol) 6000 (PEG), at pH 7.1, and centrifuged at 40000 *g* for 10 min at 4 °C. The supernatants after filtration on Sephadex G-25 (fine grade) were incubated at 30 °C in 50 mM-Hepes buffer (pH 7.1), containing 50 mM-KCl, 5 mM-MgATP, 5 mM-Fru-6-P, 17.5 mM-Glu-6-P, 2 mM-MgCl₂, 1 mM-DTT and 1 mM-P_i.

[2-³²P]Fru-2,6-P₂ was synthesized as described by El-Maghrabi *et al.* (1982). FBPase-2 activity was measured by the production of [³²P]P_i from [2-³²P]Fru-2,6-P₂. The reaction was carried out at 30 °C in 50 mM-Hepes buffer (pH 7.5), containing 50 mM-KCl, 5 mM-P_i, 2 mM-EDTA, 1 mM-DTT, 2 mM-MgCl₂, 0.1 mM-NADP⁺, 9 units of phosphoglucose isomerase/ml, 4 units of glucose-6-phosphate dehydrogenase/ml and 5 μ M-Fru-2,6-P₂ (150000 c.p.m./assay). Blanks typically did not exceed 0.2 % of the applied radioactivity.

Partial purification of brain, liver, heart and muscle PFK-2

The enzymes were purified by a two-step procedure. Tissue samples were homogenized in 5 vol. of 50 mM-Tris/phosphate, containing 100 mM-KCl, 1 mM-EGTA, 1 mM-DTT, 1 mM-benzamidine and 0.2 mM-phenylmethanesulphonyl fluoride, at pH 8.0. After centrifugation at 40000 *g* for 20 min, the supernatants were fractionated with 6 % (w/v) PEG 6000 and the resulting supernatants with 15 % (w/v) PEG 6000. The pellets (6–21 % fraction), dissolved in the homogenizing medium, were applied to a Mono Q column and eluted with a linear gradient of 0–500 mM-NaCl in the same medium. Fractions containing PFK-2 activity were pooled and stored at –80 °C in 20 % (v/v) glycerol.

Treatment of PFK-2 with the catalytic subunit of cyclic AMP-dependent protein kinase and protein kinase C

Purified PFK-2 from brain, heart, liver or muscle was incubated at 30 °C for 20 min with the catalytic subunit of cyclic AMP-dependent protein kinase (1 m-unit/ml) in a medium containing 100 mM-Hepes (pH 7.1), 1 mM-DTT, 0.1 % BSA and 1 mM-MgATP. For the protein kinase C assay, enzymes were pre-incubated for 20 min at 30 °C with 1 unit of immobilized alkaline phosphatase. Afterwards, PFK-2 enzymes were incubated for 20 min at 30 °C in the presence or absence of protein kinase C (1 m-unit/ml) in 0.1 ml of a medium containing 100 mM-Hepes (pH 7.1), 5 mM-MgCl₂, 2 mM-DTT, 5 mM-P_i, 1 mM-MgATP,

2 mM-CaCl₂, 50 μ g of phosphatidylserine/ml and 2 μ g of diolein/ml. In both cases, samples (40 μ l) were assayed for PFK-2 activity as described above at either 5 mM-Fru-6-P and 5 mM-MgATP (saturating conditions) or 0.1 mM-Fru-6-P and 0.5 mM-MgATP (subsaturating conditions).

Isolation of RNA and Northern-blot analysis

Total RNA was extracted from frozen rat tissues by the LiCl/urea method (Auffray & Rougeon, 1980). RNA was denatured and electrophoresis was performed on 1.5 % agarose gels in the presence of ethidium bromide at 1 μ g/ml. RNAs were transferred overnight to a nylon membrane in 10 × SSC (SSC = 0.15 M-NaCl/0.015 M-sodium citrate, pH 7.4). After transfer, RNA was fixed by irradiating the membrane with u.v. light for 5 min. The filters were prehybridized for 6 h at 42 °C in 50 % (v/v) formamide, 2 × PSE (PSE = 0.01 M-Pipes/0.4 M-NaCl/1 mM-EDTA at pH 6.4), 0.5 % (w/v) SDS, 5 × Denhart's solution (Denhart's solution = 0.2 g of Ficoll/1 + 0.2 g of poly(vinyl pyrrolidone)/1 + 0.2 g of BSA/1) and 0.5 mg of heat-denatured herring sperm DNA/ml. Hybridization was allowed to proceed overnight at 42 °C in the same medium, the labelled cDNA probe (1 × 10⁶–2 × 10⁶ c.p.m./ml) being added. Filters were washed twice in 2 × SSC at room temperature for 5 min and twice in 0.1 × SSC plus 0.1 % SDS at 50 °C for 30 min.

Other methods

Protein was determined (Bradford, 1976) with BSA as a standard. Protein kinase C activity was assayed with histone III-S as a substrate. One unit of enzyme activity catalyses the formation of 1 μ mol of product/min under the specified assay conditions. Statistical significance of differences was assessed by Student's unpaired *t* test.

RESULTS

Brain metabolites and enzyme activities

In order to control the starved and diabetic states of the animals we monitored the blood levels of both glucose and β -hydroxybutyrate. Glycaemia was increased 4-fold in streptozotocin-diabetic rats, whereas starved animals exhibited a continuous decrease (more than 50 % at 120 h of starvation) in blood glucose concentration. Plasma levels of β -hydroxybutyrate were increased in both situations (results not shown).

Fru-2,6-P₂ decreased similarly in both non-ketotic (Table 1) and ketotic diabetic animals (4.5 ± 0.1 nmol/g, *n* = 4). The concentration of this metabolite also decreased after lengthened ischaemia, but was not significantly modified during either the early stages of ischaemia or starvation (Table 1). Citrate increased during diabetes and starvation, but decreased during ischaemia (results not shown). No significant differences were found in the Fru-2,6-P₂ concentrations between the two different stopping methods, namely liquid N₂ (5.9 ± 0.3 nmol/g, *n* = 5) and micro-wave radiation (Table 1).

As shown in Table 1, brains from diabetic animals had significantly lower PFK-1 activities, whereas no major differences in these activities were obtained in starved animals. A fall in the activity (60 % of control), which remained after gel filtration, was also found in ischaemic animals.

PFK-2 activities obtained from control brains were about 10 times lower than those from rat liver (Bartrons *et al.*, 1983). Brain FBPase-2 (0.05 m-unit/g of tissue) represented one-tenth of the kinase activity and was not modified by 5 mM-*sn*-glycerol 3-phosphate (results not shown). No significant differences with respect to control values were observed in either kinase or

Table 1. Brain metabolites and enzyme activities

The values represent means $\pm$ S.E.M. for the number of animals in parentheses, except for Fru-2,6-P₂ where the number of animals measured was nine for diabetic and six for 120 h-starved rats. Statistically significant differences with respect to control animals are indicated by: *** P < 0.001, ** P < 0.01, * P < 0.05. N.D., not determined.

Experimental condition		Fru-2,6-P ₂ (nmol/g)	PFK-1 (units/g)	PFK-2 (m-unit/g)	FBPase-2 (m-unit/g)	PFK-2/FBPase-2 activity ratio
Control	(6)	5.8 $\pm$ 0.4	32.8 $\pm$ 1.0	0.50 $\pm$ 0.05	0.05 $\pm$ 0.01	11.7 $\pm$ 0.6
Diabetes	(4)	4.7 $\pm$ 0.2**	26.5 $\pm$ 1.3**	0.57 $\pm$ 0.08	0.08 $\pm$ 0.03	9.6 $\pm$ 2.8
120 h starvation	(4)	6.0 $\pm$ 0.5	34.8 $\pm$ 1.4	0.56 $\pm$ 0.06	0.07 $\pm$ 0.02	11.1 $\pm$ 4.5
Ischaemia (1 min)	(5)	5.7 $\pm$ 0.4	34.1 $\pm$ 2.3	0.55 $\pm$ 0.07	N.D.	N.D.
Ischaemia (5 min)	(4)	4.7 $\pm$ 0.3*	30.5 $\pm$ 0.7	0.46 $\pm$ 0.05	N.D.	N.D.
Ischaemia (10 min)	(4)	3.1 $\pm$ 0.5**	21.7 $\pm$ 1.8***	0.43 $\pm$ 0.11	0.04 $\pm$ 0.01	11.0 $\pm$ 2.6

Fig. 1. Elution profiles of (a) brain, (b) heart, (c) liver and (d) muscle PFK-2 from the Mono Q column

PEG fractions (6–21 %) of rat tissues were applied to the Mono Q column (1 ml). Fractions of volume 0.5 ml were collected after addition of the specific elution buffer. Pooled fractions contained 0.55, 0.30, 3.4 and 0.53 m-unit/mg of protein for brain, heart, liver and muscle respectively. Another two experiments were done with similar results.

phosphatase activities in streptozotocin-diabetic or starved animals. A significant decrease in PFK-2 and an increase in FBPase-2 activities were found after ischaemia, giving a decreased activity ratio. These activity changes disappeared after filtration on Sephadex G-25 (fine grade).

Properties of brain PFK-2

To characterize the main properties of brain PFK-2, the enzyme was partially purified as described above. Rat liver, muscle and heart PFK-2, the kinetic properties of which are well known, were also purified in parallel with the brain enzyme. The elution profile from the Mono Q column (Fig. 1) shows that liver

Fig. 2. Effects of *sn*-glycerol 3-phosphate and citrate on brain, heart, liver and muscle PFK-2

Purified brain, heart, liver and muscle PFK-2 were assayed in 50 mM-Hepes/100 mM-KCl/20 mM-KF/1 mM-DTT in the presence of 0.1 mM-Fru-6-P, 0.5 mM-MgATP and the indicated concentrations of *sn*-glycerol 3-phosphate or citrate, at pH 7.1. The specific activities corresponding to 100 % values were 0.063 for brain (□), 0.029 for heart (○), 0.87 for liver (■) and 0.087 m-unit/mg of protein for muscle (●). The values are means of two separate experiments.

and muscle PFK-2 were eluted in two peaks at about 0.4 M-NaCl. In contrast, brain PFK-2 was eluted at 0.2 M-NaCl, displaying a similar behaviour to its heart counterpart. Both PFK-2 and FBPase-2 activities were eluted in the same fractions from the Mono Q column. Purified brain PFK-2/FBPase-2 showed an activity ratio of 70, similar to that of the purified bovine heart isoenzyme (Rider & Hue, 1986). Purified brain and liver PFK-2 also differed in their response to *sn*-glycerol 3-phosphate and citrate at concentrations within the physiological range. Whereas the activity of the liver enzyme was markedly inhibited by *sn*-glycerol 3-phosphate, the brain enzyme, similarly to the muscle or heart isoenzyme, was little affected by concentrations of up to 1 mM. Furthermore, the brain and heart PFK-2 were more sensitive to inhibition by citrate (Fig. 2).

It has been reported that liver PFK-2 is inactivated by the catalytic subunit of the cyclic AMP-dependent protein kinase, whereas enzymes from other tissues are insensitive to this modification. Table 2 shows that, whereas liver PFK-2 is inhibited by treatment with the catalytic subunit of cyclic AMP-dependent protein kinase, the brain enzyme, together with the muscle and heart counterparts, are not modified at all. In order to determine whether the absence of inactivation in brain PFK-2 could result from the fact that the enzyme was in a fully phosphorylated

Table 2. Effect of treatment of PFK-2 with the catalytic subunit of cyclic AMP-dependent protein kinase

Purified brain, heart, liver or muscle PFK-2 was incubated at 30 °C for 20 min in the presence or absence of catalytic subunit of cyclic AMP-dependent protein kinase (0.1 m-unit) in a final volume of 0.1 ml containing 100 mM-Hepes (pH 7.1), 1 mM-DTT, 0.1 % BSA and 1 mM-MgATP. PFK-2 activities were assayed as described in the Materials and methods section. The values are means of two separate experiments.

Addition	PFK-2 activity (mU/mg of protein)			
	Brain	Heart	Liver	Muscle
None	0.49	0.18	4.03	0.65
+ catalytic subunit	0.46	0.17	0.98	0.60

Fig. 3. Northern-blot analysis

Total RNA (40 µg per lane) from liver (L), muscle (M), brain (B) and heart (H) were hybridized with [³²P]cDNA^{2k1400}. Arrows point to the RNA species consistently seen. Numbers on the left correspond to the RNA markers. Data are representative of three different experiments.

form, brain PFK-2 was incubated with glucose, MgADP, hexokinase and the catalytic subunit of the cyclic AMP-dependent protein kinase to dephosphorylate the enzyme (El-Maghrabi *et al.*, 1980; Loiseau *et al.*, 1988); no activation of PFK-2 was observed.

Since brain PFK-2 seems to display similar chromatographic and kinetic properties to that of heart (Figs. 1 and 2, and Table 2) and bearing in mind that previous studies have shown that heart PFK-2 could be phosphorylated by protein kinase C (Rider & Hue, 1986; Kitamura *et al.*, 1988; Sakata & Uyeda, 1990), we have analysed the effects of protein kinase C on the PFK-2 activity of brain, heart, liver and muscle isoenzyme forms. Purified enzymes were preincubated for 20 min with 1 unit of agarose-bound alkaline phosphatase for dephosphorylation, and then incubated with protein kinase C. We found no changes in activity in any of them, at either subsaturating or saturating concentration of substrates (results not shown).

Expression of distinct mRNAs for PFK-2/FBPase-2 in rat tissues

Northern blots of total RNA from rat tissues were hybridized with probe cDNA^{2k1400} (Colosia *et al.*, 1988). All tissues studied displayed a strong signal which persisted under high-stringency conditions. Fig. 3 shows a signal with 2.1 kb RNA from liver and 1.9 kb RNA from muscle. RNA from brain and heart shows a major band of about 6.8 kb RNA and a minor band of 4 kb. The relative intensity of these signals did not change appreciably under high-stringency conditions.

DISCUSSION

Metabolic studies of brain have shown that glucose under most circumstances is the primary source of fuel (McIlwain & Bachelard, 1985). It is well known that acute modifications in substrate supplies such as those produced in starvation, diabetes or ischaemia promote changes in brain metabolism. These changes mainly affect glycolytic flux through modifications of the main regulatory enzymes.

During starvation and diabetes, ketone bodies increase appreciably and may become a significant substrate for the brain, partly replacing glucose as fuel for brain metabolism (Siesjö, 1988; Owen *et al.*, 1967; Ruderman *et al.*, 1974). It has been suggested that ketone-body oxidation will restrict the rate of glucose utilization through citrate inhibition of PFK-1 during periods of carbohydrate deprivation, starvation or diabetes (Owen *et al.*, 1967; Ruderman *et al.*, 1974; Newsholme *et al.*, 1977). The results now reported show that, whereas Fru-2,6-*P*₂ and PFK-1 are decreased during diabetes, neither of them changes significantly during starvation. On the other hand, citrate is increased in both situations. The decrease in Fru-2,6-*P*₂ levels observed in diabetic animals is probably a consequence of the inhibition of PFK-2 by citrate (see Fig. 2), since no changes in either kinase or phosphatase activities were found. Brain PFK-1 (Newsholme *et al.*, 1977; Dunaway, 1983; Foe & Kemp, 1985; Vora *et al.*, 1985) and PFK-2 (Fig. 2) isoenzymes are very sensitive to citrate inhibition, which, in addition to the fall in Fru-2,6-*P*₂ levels, could explain the decreased rate of glycolysis in rat brain when ketone bodies are oxidized (Ruderman *et al.*, 1974; Newsholme *et al.*, 1977). The PFK-1 and PFK-2 isoenzymes that are more sensitive to citrate inhibition are from the same tissues (brain and heart).

During ischaemia there is rapid deterioration in the energy charge, and the tissue levels of both glycolytic substrates and intermediates are decreased (Lowry *et al.*, 1964). Pauwels & Trouet (1984) found no predominant role of Fru-2,6-*P*₂ at the onset of anaerobic glycolysis in various types of cultivated brain cells, concluding that it seemed not to be responsible for immediate changes in glycolytic flux. Yamamoto *et al.* (1990) reported that, in the early stages of ischaemia, Fru-2,6-*P*₂ content increases transiently in parallel with Fru-1,6-*P*₂ and lactate formation, decreasing steadily after lengthened ischaemia without changes in the PFK-2 activity. These results suggest that Fru-2,6-*P*₂ plays a key role in the compensatory acceleration of glycolysis under ischaemic conditions *in vivo*. In contrast, Ogushi *et al.* (1990) found that there was no change in Fru-2,6-*P*₂ during initiation of rapid glycolysis in the brain. Our results show that the metabolite content was similarly unchanged in the first 1 min of ischaemia, but decreased thereafter (Table 1). The variations in the metabolite concentrations (Fru-6-*P*, AMP and IMP), the large fall in ATP levels and the change in the activity ratio of PFK-2/FBPase-2 induced by allosteric modulators, would explain the decrease in Fru-2,6-*P*₂ content. A good correlation is

obtained for the two metabolites below $1\text{ }\mu\text{mol}$ of ATP/g ($r = 0.82$; $P < 0.0001$) (results not shown).

Glu-1,6- P_2 has also been identified as a modulator of the activities of all three of the potentially rate-limiting enzymes of glycolysis (Beitner, 1985). The level of Glu-1,6- P_2 is not modified during starvation or diabetes, and is more affected than Fru-2,6- P_2 during ischaemia (results not shown) (Passonneau *et al.*, 1969), probably as a result of the concomitant rise in IMP and Ca^{2+} , both activators of glucose-1,6-bisphosphatase (Wakelam & Emmerich, 1982; Guha & Rose, 1983). The activation of PFK-1 that occurs during the early stages of ischaemia takes place when Glu-1,6- P_2 concentration is falling, and the activation seems best explained by other factors such as AMP, Fru-2,6- P_2 , P_i or ribose 1,5-bisphosphate, which are much better activators of PFK-1 than is Glu-1,6- P_2 (Rose & Warms, 1974; Yip *et al.*, 1988; Ogushi *et al.*, 1990).

Because the brain is not subjected to the tremendous differences in energy demands that are observed in other tissues, it maintains a fairly constant high rate of glucose metabolism. Similarly to glycogen (Nahas & Abdul-Ghani, 1989) and Glu-1,6- P_2 , the concentration of Fru-2,6- P_2 seems to be remarkably constant under a wide variety of physiological and experimental conditions. Its levels probably have an essential role in the basal activation of brain PFK-1.

Rat brain PFK-2/FBPase-2 also seems to remain essentially constant in all the experimental conditions studied. The PFK-2 and FBPase-2 activities were co-eluted in the same fractions, suggesting that the enzyme is also bifunctional in this tissue, which agrees with El-Maghrabi *et al.* (1986). Its FBPase-2 activity is 70 times smaller than the PFK-2 activity, accounting probably for the relatively high levels of the metabolite, although the enzyme activity is 10 times lower than in the liver. Chromatographic results showed that brain PFK-2/FBPase-2 has a molecular form with a net charge similar to that of the heart tissue. The brain enzyme is not inhibited by *sn*-glycerol 3-phosphate and is more sensitive to citrate than the liver or muscle isoenzyme. Heart PFK-2 is phosphorylated by protein kinase C to the extent of about 1 mol/mol of enzyme subunit, whereas liver PFK-2 is a relatively poor substrate. This phosphorylation does not alter the activity of the enzyme (Rider & Hue, 1986). In contrast, Kitamura *et al.* (1988) have shown that the purified heart PFK-2/FBPase-2 can be slightly activated by phosphorylation through cyclic AMP-dependent protein kinase or protein kinase C. The phosphorylation sites of the heart isoenzyme were located near the C-terminus (Sakata & Uyeda, 1990), whereas the phosphorylation site of the liver isoenzyme is known to be located near the N-terminus (Lively *et al.*, 1988; Darville *et al.*, 1989). The enzymic activity from brain PFK-2, similarly to that of heart or muscle (Rider & Hue, 1986; Rider, 1987), is not modified on incubation with cyclic AMP-dependent protein kinase or with Ca^{2+} - and phospholipid-dependent protein kinase (protein kinase C).

RNA from brain and heart shows a major band of about 6.8 kb and a minor one of 4 kb, agreeing with previous results (Sakata & Uyeda, 1990; Crepin *et al.*, 1989). Total RNA used for Northern-blot assays may contain precursor as well as mature RNA. This fact might explain the two species detected in brain and heart RNA. However, the shorter RNA detected is 4 kb, which is too big to code the 530 amino acids of bovine heart PFK-2/FBPase-2 (Sakata & Uyeda, 1990). It would suggest that a polypeptide coding for the enzyme could be processed post-translationally, or that cDNA^{2k1400} strongly cross-hybridizes with other related transcripts present in these tissues.

All these properties support the view that the brain contains a different isoenzyme form from that of liver or muscle and that it is probably related to the isoenzyme from heart.

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