Bovine Brain 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase
EVIDENCE FOR A NEURAL-SPECIFIC ISOZYME*

(Received for publication, March 23, 1992)

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Bovine brain 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase was purified to homogeneity and characterized. This bifunctional enzyme is a homodimer with a subunit molecular weight of 120,000, which is twice that of all other known bifunctional enzyme isozymes. The kinase/bisphosphatase activity ratio was 3.0. The $K_{\text{m}}$ values for fructose 6-phosphate and ATP of the 6-phosphofructo-2-kinase were 27 and 55 $\mu$M, respectively. The $K_{\text{m}}$ for fructose 2,6-bisphosphate and the $K_p$ for fructose 6-phosphate for the bisphosphatase were 70 and 20 $\mu$M, respectively. Physiologic concentrations of citrate had reciprocal effects on the enzyme's activities, i.e. inhibiting the kinase ($K_p$ of 35 $\mu$M) and activating the bisphosphatase ($K_p$ of 16 $\mu$M). Phosphorylation of the brain enzyme was catalyzed by the cyclic AMP-dependent protein kinase with a stoichiometry of 0.9 mol of phosphate/mol of subunit and at a rate similar to that seen with the liver isozyme. In contrast to the liver isozyme, the kinetic properties of the brain enzyme were unaffected by cyclic AMP-dependent protein kinase phosphorylation, and also was not a substrate for protein kinase C. The brain isozyme formed a labeled phosphoenzyme intermediate and cross-reacted with antibodies raised against the liver isozyme. However, the NH2-terminal amino acid sequence of a peptide generated by cyanogen bromide cleavage of the enzyme had no identity with any known bifunctional enzyme sequences. These results indicate that a novel isozyme, which is related to other 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase isoforms, is expressed specifically in neural tissues.

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* This work was supported by Grant DGICYT PB91/0239 from Dirección General de Investigación Científica y Técnica and by National Institutes of Health Grant DK-38354 (to S. L. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ Recipient of a research fellowship (FPI) from the Ministry of Education of Spain.

§ The abbreviations used are: Fru-2,6-P$_2$, fructose 2,6-bisphosphate; Fru-6-P, fructose 6-phosphate; 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); CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; PEG, polyethylene glycol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGTGA, [ethylenebis(oxycetylenenitri1o)tetraacetic acid]; bp, base pair(s).

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and breakdown are catalyzed by 6-phosphofructo-2-kinase (EC 2.7.1.105) and fructose-2,6-bisphosphatase (EC 3.1.3.46), respectively (1–3). These two activities belong to separate domains of each subunit of the same homodimeric protein. This bifunctional enzyme integrates a number of metabolic and hormonal signals by means of allosteric effectors and phosphorylation/dephosphorylation processes and by its transcription rate (1–9).

Several mammalian 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) isozymes have been described whose expression differs depending on the tissue (1–3, 10–14). The liver “L” and muscle “M” isozymes derive from a 55-kilobase gene by alternative splicing from two promoters (15). Its expression is controlled by ubiquitous and tissue-specific transcription factors (16) and hormones (6–7), the most important being glucocorticoids (5, 7). The heart “H” type contains a COOH-terminal extension that includes a sequence that codes for a protein kinase C and cyclic AMP-dependent protein kinase (PKA) phosphorylation sites (17).

Fru-2,6-P$_2$ is present in various types of cultivated brain cells at a concentration similar to that in hepatocytes (18), and it is also a positive allosteric effector of brain PFK-1 (19–20). The bisphosphorylated metabolite increases transiently, in parallel with fructose 1,6-bisphosphate and fructose 6-phosphate, during early stages of ischemia (21). It also decreases during hypoglycemia following the fall in fructose 6-phosphate and increases to normal levels after glucose administration (22). In the diabetes state, Fru-2,6-P$_2$ levels are decreased permitting the glucose-sparing effects of ketone bodies on brain glycolysis (23). However, all the changes in brain Fru-2,6-P$_2$ are quantitatively low, suggesting that in brain tissue Fru-2,6-P$_2$ remains remarkably constant under a wide variety of experimental conditions, suggesting that it plays a permissive role in cerebral glycolysis, by maintaining PFK-1 in an active state.

As a first step in understanding how brain Fru-2,6-P$_2$ levels are regulated, we report the isolation and characterization of a bovine brain PFK-2/FBPase-2 isozyme which has unique kinetic and structural properties compared to other known mammalian isozymes, and which is probably encoded by a heretofore undiscovered gene.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP (3000 C/mmol) was from Amersham, Mono-Q and Superose 12 HR columns and Blue Sepharose were from Pharmacia LKB Biotechnolog Inc. Enzymes and other biochemical reagents were purchased from either Boehringer or Sigma. Pro-Blot transfer membranes were from Applied Biosystems. Purified protein kinase C from rat brain was kindly given by Dr. E. Rozengurt (Imperial Cancer Research Fund, London).

Enzyme Assays—PFK-2 activity was determined by a modification of the method described by Bartrons et al. (24). Samples were incu-
bated at 30 °C in 50 mM Hepes buffer (pH 7.1) containing 50 mM KCl, 1 mM dithiothreitol, 1 mM PEG, and, unless otherwise indicated, 5 mM ATP, 5 mM fructose 6-phosphate (Fru-6-P), and 17.5 mM glucose 6-phosphate.

FPK-2ase activity was measured by the release of 32P from [2-32P]-Fru-2,6-P2 as described by Ventura et al. (23). [2-32P]-Fru-2,6-P2 was synthesized according to El-Maghrabi et al. (25). Western Blot Analysis—Immunoblot analysis was performed essentially as described by Burnette (26) with a 1:200 dilution of the polyclonal antibody raised against rat liver PFK-S/FBPase-2 (27).

RESULTS

Purification of 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase—Bovine brains were obtained from a local slaughterhouse, maintained in ice, and used immediately. Cortex from dissected brains (700 g) was trimmed away from the meninges and blood vessels and homogenized in 3 volumes of ice-cold 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 2 mM EGTA, 2 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin (buffer B) and clarified by centrifugation at 15,000 × g for 15 min. The enzyme was then applied to a Blue Sepharose CL6B column (1.8 × 35 cm) which had been equilibrated with buffer B. The column was washed overnight with buffer B until the eluate was free of protein (monitored by A280). PFK-2/FPK-2ase was eluted (5–10 mM ATP-Mg) with a linear gradient of ATP-Mg (0–15 mM) in buffer B. The fractions containing PFK-2 activity were pooled and ultrafiltered to approximately 3 ml with an Amicon concentrator equipped with a YM-10 filter. The concentrated enzyme was diluted 4-fold and applied to a Mono-Q column equilibrated with 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 2 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin, and 10% (v/v) glycerol (buffer C). After washing with the equilibration buffer a continuous KCl gradient (0–300 mM) made in buffer C was applied. PFK-2/FPK-2ase eluted at 0.2 mM KCl. The fractions containing the peak activity were pooled, diluted 2-fold, and reapplied in the same column.

The enzyme eluted as a single peak with a flatter gradient made in the same buffer (Fig. 1). The peak fractions containing the enzyme were stored frozen at −80 °C in 20% (v/v) glycerol.

This purification method permitted the isolation of bovine brain PFK-2/FPK-2ase with an average 10% yield. The procedure took less than 4 days to complete and led to a 15,000-fold purification (Table I). No other significant Fru-2,6-P* synthetizing activities were found during the purification procedure. The purified fractions displayed both PFK-2 and FPK-2 activities indicating that the bovine brain enzyme is bifunctional with specific activities of 90 milliunits/mg of protein.

FIG. 1. Elution profile from Mono-Q column and SDS-PAGE of bovine brain PFK-2/FPK-2ase. PFK-2/FPK-2ase from bovine brain was reapplied into a Mono-Q column as described under “Results.” The enzyme was eluted between 19 and 21 min. A fraction of the peak was subjected to gel electrophoresis in an 8% acrylamide gel. The position of molecular mass standards is indicated by the arrows.

| Steps | Protein | Purification Yield |
|-------|---------|--------------------|
| Extract | 25002 mg | 1 100 |
| 6–21% PEG | 4242 mg | 82.4 0.019 3.2 56 |
| Blue Sepharose | 3.44 mg | 48.4 14.1 2345 32.9 |
| Mono-Q | 0.71 mg | 31.6 44.5 7417 21.5 |
| Mono-Q reapplication | 0.15 mg | 13.9 92.7 15450 9.5 |
protein for the kinase and 29 milliunits/mg of protein for the bisphosphatase.

Molecular Weights—The $M_0$ of the subunit was determined to be 120,000 by SDS-PAGE (Figs. 1 and 2). The molecular weight of the native enzyme was estimated to be 225,000 by molecular filtration on a Superose 12 HR column in a 200 mM phosphate buffer, pH 7.0. These data are indicative of a homodimeric structure for the brain bifunctional enzyme.

Western Blot and Phosphoenzyme Assays of PFK-2/ FBPase-2 from Brain—Purified brain and liver enzymes were subjected to Western blotting after labeling with Fru-2,6-[2-32P]P2 (Fig. 2). The Western blot showed that anti-liver PFK-2/FBPase-2 polyclonal antibodies cross-reacted with the brain form indicating that they share some antigenic relatedness. In addition, brain PFK-2/FBPase-2 formed a labeled phosphoenzyme intermediate upon brief incubation with Fru-2,6-[2-32P]P2, suggesting that the brain bisphosphatase reaction involves a phosphohistidine intermediate as does the other mammalian isozymes (3).

Kinetic Properties—Several kinetic properties of bovine brain PFK-2/FBPase-2 were determined (Table II). The $K_m$ values for Fru-6-P and ATP are in the same range as those determined for the heart and muscle isozymes (10–11, 30). The $K_m$ value for Fru-6-P (27 μM) is lower than that of the liver form (1–3). The bisphosphatase has a high $K_m$ value for Fru-2,6-P2 (70 μM) and also showed a high sensitivity to noncompetitive inhibition by Fru-6-P (86% inhibition at 100 μM Fru-6-P). Citrate had reciprocal effects on both activities, inhibiting kinase and activating bisphosphatase. Citrate reduced the $K_m$ for Fru-2,6-P2 to 50% without changing the $V_{\text{max}}$ value of the bisphosphatase activity. Other known modulators of liver PFK-2/FBPase-2 such as glycerol 3-phosphate or phospho(enol)pyruvate (1–3) (up to 5 mM each) did not modify either kinase or bisphosphatase activities. ATP also had no effect on the bisphosphatase activity.

Phosphorylation of Brain PFK-2/FBPase-2 by Cyclic AMP-dependent Protein Kinase and Protein Kinase C—To analyze the nature of brain PFK-2/FBPase-2 the purified enzyme was tested, in parallel with the liver isozyme, as a substrate for the catalytic subunit of cyclic AMP-dependent protein kinase and protein kinase C. These two protein kinases have been shown to differentially phosphorylate PFK-2/FBPase-2 isoforms (1–3, 31). As shown in Fig. 3, the catalytic subunit of cyclic AMP-dependent protein kinase catalyzed the phosphorylation of both the brain and liver isozymes. When similar concentrations of pure bovine brain or rat liver isozymes were incubated with the catalytic subunit of cyclic AMP-dependent protein kinase, their phosphorylation was catalyzed at similar rates (Fig. 3). The stoichiometries of phosphorylation were 0.9 mol of phosphate/mol subunit for the brain enzyme and 0.8 mol of phosphate/mol subunit for the liver enzyme. These results indicate a ratio close to 1 suggesting that brain PFK-2/FBPase-2 has a single phosphorylation site. However, unlike the heart and testes isozymes which are substrates for protein kinase C, the brain enzyme was not phosphorylated by protein kinase C (data not shown). In agreement with our previous results (23), incubation with the catalytic subunit of cyclic AMP-dependent protein kinase did not alter the $K_m$ for Fru-6-P or $V_{\text{max}}$ of bovine brain PFK-2 at pH 7.1, whereas it did modify the activity of the liver isozyme (23). In order to determine whether the absence of activation could result from the fact that the enzyme remained in a phosphorylated form, the enzyme was incubated with glucose, ADP-Mg, hexokinase, and the catalytic subunit of cyclic AMP-dependent protein kinase in order to remove phosphate specifically from the enzyme by a reversal of the cyclic AMP-dependent protein kinase reaction (32). However, no change in activity was observed. It was concluded that cyclic AMP-dependent protein kinase catalyzed phosphorylation of the brain enzyme.

Figure 2. SDS-PAGE, Western blot, and fructose 2,6-bisphosphate labeling of brain PFK-2/FBPase-2. The left panel is the Coomassie Blue stain of purified brain PFK-2/FBPase-2. The center panel is the immunoblot of brain and liver enzymes that were performed as described under “Experimental Procedures.” The right panel shows SDS-PAGE electrophoresis of 32P-labeled brain enzyme with Fru-2,6-[2-32P]P2.

Table II: Kinetic constants of bovine brain PFK-2/FBPase-2

| Enzyme      | $V_{\text{max}}$ (mU/mg) | $K_m$ (μM) | $K_m$ (μM) | $K_m$ (μM) |
|-------------|--------------------------|------------|------------|------------|
| PFK-2       | 90                       | 55         | 27         | 35         |
| FBPase-2    | 29                       | 70         | 20         | 16         |

Figure 3. Phosphorylation of bovine brain and rat liver PFK-2/FBPase-2 by cyclic AMP-dependent protein kinase. PFK-2/FBPase-2 from (B) brain (0.5 μM) and (L) liver (1 μM) were incubated in the presence of 2 milliunits/ml catalytic subunit of cyclic AMP-dependent protein kinase as described under “Experimental Procedures.” At the indicated times, aliquots (40 μl) were removed, and the amount of [32P]phosphate incorporated on bovine brain (●) or rat liver (○) was determined. The values are the means ± S.E. of three separate experiments. The phosphorylated enzymes (20 min) were subjected to gel electrophoresis and autoradiographed.
does not affect the activity of the enzyme.

**Cyanogen Bromide Cleavage and Amino Acid Sequencing**—In order to obtain further structural information about the nature of the enzyme we sequenced the native protein after alkylation with 4-vinylpyridine. The results showed that the NH₂ terminus of the protein was blocked to Edman degradation. PKF-2/FBPase-2 (5-10 µg) was then partially digested with CNBr after alkylation as described under “Experimental Procedures.” The cleaved peptides were subjected to SDS-PAGE in 15% acrylamide, 0.75% bisacrylamide gels. The gels were electroblotted and the membranes were stained with Coomassie Blue and washed extensively with water. Three major peptides were detected with molecular weights of 36,000, 32,000, and 22,000. The blotted 36-kDa peptide (50 pmol) was sequenced directly from the membrane with certainty to its 10th residue. The sequence was Glu-Val-Asn-Gln-His-Leu-Lys-Gly-Glu-Tyr. This sequence has no significant homology to any of the known sequences of other mammalian PKF-2/FBPase-2s (13, 15, 17, 33-34).

**Discussion**

We report here a procedure for the isolation of PKF-2/FBPase-2 from bovine brain resulting in a 15,000-fold purification. The enzyme was pure as judged by SDS-PAGE and both Coomassie Blue or silver staining and had an average native molecular weight of 225,000 corresponding to an homodimer of two M, 120,000 subunits. This subunit molecular weight is approximately twice that of all known mammalian bifunctional enzyme forms, since the liver, skeletal muscle, and heart forms have molecular masses of 55, 64, and 58 kDa, respectively (1-3, 8-11, 13). The homogeneous enzyme exhibited both activities, and the kinase/bisphosphatase activity ratio remained constant through all purification steps after the PEG fractionation (data not shown). The specific kinase activity was 90 and 29 millimolars/mg of protein for the bisphosphatase. No other significant brain forms of PKF-2/FBPase-2 were found during the purification procedure, whether cortex or whole brain was used as the tissue source. Based on the above findings, the brain form represents a unique isozyme but is bifunctional like all other mammalian isozymes.

Different mammalian tissue-specific PKF-2/FBPase-2 isozymes have been described. The liver and muscle isozymes differ only in the first exon, sharing the other 13 exons (15). The heart type is encoded by another gene (35) that includes both activities, and the kinase/bisphosphatase activity ratio remained constant through all purification steps after the PEG fractionation (data not shown). The specific kinase activity was 90 and 29 millimolars/mg of protein for the bisphosphatase. No other significant brain forms of PKF-2/FBPase-2 were found during the purification procedure, whether cortex or whole brain was used as the tissue source. Based on the above findings, the brain form represents a unique isozyme but is bifunctional like all other mammalian isozymes.

The concentration of Fru-2,6-P₂ in mammalian tissues is controlled by the relative activities of PKF-2 and FBPase-2. The kinase/bisphosphatase activity ratio is close to 3 for the brain enzyme when both activities are determined under Vₘₐₓ conditions. However, the bisphosphatase has a very high Kₛ for its substrate (70 µM) compared to that of the liver enzyme (0.1 µM). This fact suggests that under physiological conditions the high kinase/bisphosphatase ratio accounts for the high concentration of the bisphosphorylated metabolite found in brain. Work is in progress to define the structural basis for the differences between the larger brain form and the smaller mammalian forms and to ascertain whether the 120-kDa brain isozyme is evolutionarily related to the 93-kDa yeast enzyme.

An increase of Fru-2,6-P₂ would result in a parallel increase of Fru-2,6-P₂, by virtue of increased kinase activity as well as decreased bisphosphatase activity via noncompetitive product inhibition (Table II). In contrast, any diminution of Fru-6-P concentration would result in lower levels of the bisphosphorylated metabolite, which would decrease the net glycolytic flux. This scenario is seen during brain ischemia or hypoglycemia (21-23). These considerations do not apply to the other substrate of the kinase activity, since the Kₛ for ATP is substantially lower than the steady state tissue concentration of ATP (2-3 mM), so the enzyme would be fully saturated under normal conditions. Other regulatory metabolites such as glyceral 3-phosphate or phosphoenolpyruvate which act as allosteric effectors on liver PKF-2/FBPase-2 (1-3) have no effect on the brain enzyme (23 and results not shown). However, citrate has a reciprocal effect on both activities. Not only does it inhibit the kinase, but it also activates the bisphosphatase by decreasing the Kₛ for its substrate (2-fold activation at 5 mM Fru-2,6-P₂). Citrate, which is increased in diabetic animals (23, 38), may mediate the glucose sparing effect of ketone bodies on brain glycolysis by its effects on PKF-1 (39) and PKF-2 (present paper). When available, ketone body metabolism in brain increases citrate levels which

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1. F. Ventura, J. L. Rosa, S. Ambrosio, S. J. Pilkis, and R. Bartrons, manuscript in preparation.
inhibit directly brain PFK-1 and also decrease Fru-2,6-P₂ concentration.

Phosphorylation/dephosphorylation mechanisms that control the relative kinase/bisphosphatase activity ratio have been demonstrated for the liver isozyme (1–3). The brain enzyme was phosphorylated by cAMP-dependent protein kinase but, unlike the heart and testis forms, it was not phosphorylated by protein kinase C (13, 31). In its phosphorylation pattern, the brain PFK-2/FBPase-2 is similar to the liver isozyme, even though the significance of its phosphorylation is unclear since the phosphorylated enzyme showed no changes in kinetic properties (23). Therefore, the control of PFK-2/FBPase-2 activities depends mainly on the changes of substrates and allosteric modulators. In addition, no changes in activity have been detected in several physiological conditions (21–23). Since the brain enzyme has only a single phosphorylation site, its molecular weight is approximately double that of the other mammalian isozymes, it is unlikely that the brain enzyme arose via gene duplication. It is possible that an additional NH₂- or COOH-terminal sequence prevents the transmission of the phosphorylation site signal to the kinase and bisphosphatase domains. It seems reasonable to postulate that the brain isozyme has evolved to meet the metabolic exigencies of that tissue, that additional polypeptide sequence in this form ensures regulation of brain Fru-2,6-P₂ concentration by substrates and effectors of the enzyme, and that covalent modification induced-activity changes are inexpedient in this purely glycolytic tissue.

Acknowledgments—We thank A. Tauler, J. Rodrigues, J. Gil, M. Dalmou, R. El-Maghrahi, and A. Lange for their help and for many valuable suggestions during the course of this work. The skillful technical assistance of C. Ortufio and T. Fisher is also acknowledged.

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