THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 267, No. 25, Issue of September 5, pp. 17939-17943, 1992
Printed in U.S.A.


# 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 $K_m$ values for fructose 6-phosphate and ATP of the 6-phosphofructo-2-kinase were 27 and 55 μM, respectively. The $K_m$ for fructose 2,6-bisphosphate and the $K_i$ for fructose 6-phosphate for the bisphosphatase were 70 and 20 μM, respectively. Physiologic concentrations of citrate had reciprocal effects on the enzyme's activities, *i.e.* inhibiting the kinase ($K_i$ of 35 μM) and activating the bisphosphatase ($K_a$ of 16 μM). 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 $NH_2$-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 isozymes, is expressed specifically in neural tissues.**

Fructose 2,6-bisphosphate (Fru-2,6-$P_2$)[1] is the most powerful activator of 6-phosphofructo-1-kinase (EC 2.7.1.11) (PFK-1), a key regulatory enzyme of glycolysis. Its synthesis 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*—[$\gamma$-$^{32}$P]ATP (3000 $C_i$/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-

* This work was supported by Grant DGICYT PB91/0239 from Dirección General de Investigación Científica y Tecnica and by National Institutes of Health Grant DK-38354 (to S. 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.

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

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[1] The abbreviations used are: Fru-2,6-$P_2$, fructose 2,6-bisphosphate; Fru-6-P, fructose-6-P; 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, polyethyleneglycol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGTA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid)]; bp, base pair(s).

bated at 30 °C in 50 mM Hepes buffer (pH 7.1) containing 50 mM KCl, 1 mM ditiothreitol, 1 mM $P_i$, and, unless otherwise indicated, 5 mM ATP, 5 mM fructose 6-phosphate (Fru-6-P), and 17.5 mM glucose 6-phosphate.

FBPase-2 activity was measured by the release of $^{32}P_i$ from [2-$^{32}$P] Fru-2,6-$P_2$ as described by Ventura *et al.* (23). [2-$^{32}$P]Fru-2,6-$P_2$ was synthesized according to El-Maghrabi *et al.* (25).

*Western Blot Analysis*—Immunoblot analysis were performed essentially as described by Burnette (26) with a 1:200 dilution of the polyclonal antibody raised against rat liver PFK-2/FBPase-2 (27). Bound antibodies were detected by incubation with $^{125}$I-labeled protein A (1–2 × $10^6$ cpm/ml) for 30 min and, after washing, exposed to x-ray film.

*Phosphoenzyme Assay*—Phosphoenzyme autoradiograms were obtained by a modification of El-Maghrabi *et al.* (27). Briefly, purified bovine brain PFK-2/FBPase-2 (0.5–2 μg) was incubated at room temperature with 1 μM Fru-2,6-[2-$^{32}$P]$P_2$ (1–2 × $10^6$ cpm/assay) in 50 mM Hepes buffer (pH 7.5), 50 mM KCl, 2 mM $MgCl_2$, 1 mM ditiothreitol. The reaction was stopped after 15 s by addition of 2 volumes of ice-cold 10% trichloroacetic acid. After centrifuging, the pellet was washed with 1% HCl in acetone and centrifuged again. The pellet was then redissolved in 25 μl of 0.1 M $NH_4HCO_3$ (pH 9.0), and the sample was analyzed by SDS-PAGE in a 8% gel.

*Phosphorylation of PFK-2/FBPase-2 by Cyclic AMP-dependent Protein Kinase and Protein Kinase C*—Brain PFK-2/FBPase-2 was incubated for 20 min at 30 °C with the purified catalytic subunit of cyclic AMP-dependent protein kinase (2 milliunits/ml) in a medium containing 100 mM Hepes (pH 7.1), 5 mM $MgCl_2$, 1 mM ditiothreitol, and 0.5 mM [γ-$^{32}$P]ATP-Mg (1–2 × $10^6$ cpm/nmol). For the protein kinase C assay, PFK-2/FBPase-2 was incubated for 20 min at 30 °C with protein kinase C (2 milliunits/ml) in a medium containing 100 mM Hepes (pH 7.1), 5 mM $MgCl_2$, 1 mM ditiothreitol, 5 mM $P_i$, 0.5 mM [γ-$^{32}$P]ATP-Mg (1 × $10^6$ cpm/nmol), 2 mM $CaCl_2$, 50 μg of phosphatidylserine/ml, and 2 μg of diolein/ml. After the incubation, phosphorylated samples were subjected to SDS-PAGE. The gel was then dried and exposed to x-ray film.

*Cyanogen Bromide Digestion and Amino Acid Sequencing*—Blotted PFK-2/FBPase-2 was suspended in 100 μl of 70% formic acid, and CNBr was added to a final concentration of 5 mg/ml. The tube was flushed with nitrogen and incubated in the dark for 24 h at room temperature. The incubation mixture was diluted 10 times and dialyzed overnight against 2 mM β-mercaptoethanol. After dialysis, digested PFK-2/FBPase-2 was subjected to SDS-PAGE in a 15% acrylamide, 0.75% bisacrylamide gel. The gel was transferred to a Pro-Blot membrane (Applied Biosystems) for 45 min at 70 V in 10 mM CAPS, 10% methanol (v/v), pH 11. The blotted membrane was stained with Coomassie Blue, and bands of interest were excised and washed extensively with water. The amino acid sequence was determined in a liquid-phase sequencer (Applied Biosystems 470-A protein sequencer).

*Other Methods*—SDS-PAGE was carried out in a 8% gels according to the procedure of Laemmli (28). Protein was determined by the method of Bradford (29) using bovine serum albumin as a standard. Protein kinase C activity was assayed with histone III-S as a substrate. One unit of enzyme activity catalyzes the formation of 1 μmol of product per min under the specified assay conditions.

## 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 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 ditiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin, 0.1 mM Fru-6-P, 0.3 mM glucose 6-phosphate, and 3% (w/v) polyethylene glycol 6000 (PEG 6000). The homogenate was centrifuged at 15,000 × *g* for 20 min. 3% (w/v) PEG 6000 was added to the supernatant and after 10 min it was subjected to centrifugation, and the pellet was discarded. 15% PEG 6000 was added to the supernatant, the pH was adjusted to 6.5 with HCl, and after standing for 10 min it was centrifuged at 15000 × *g* for 20 min. The pellet (6–21% PEG 6000) was redissolved in 300 ml of ice-cold 50 mM Tris-HCl (pH 8.0), 400 mM KCl, 2 mM ditiothreitol, 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 $A_{280}$). PFK-2/FBPase-2 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 ditiothreitol, 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/FBPase-2 eluted at 0.2 M 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/FBPase-2 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-$P_2$ synthesizing activities were found during the purification procedure. The purified fractions displayed both PFK-2 and FBPase-2 activities indicating that the bovine brain enzyme is bifunctional with specific activities of 90 milliunits/mg of

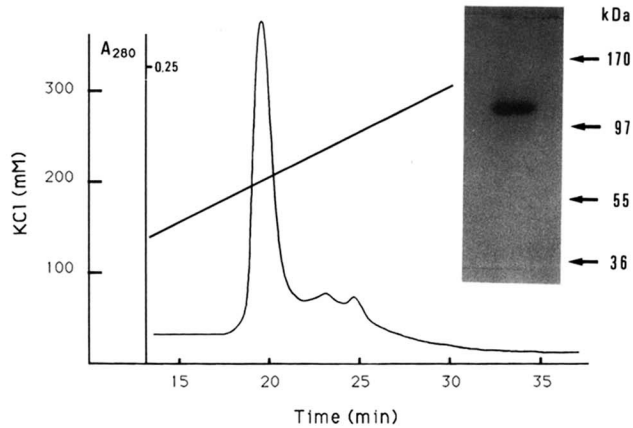


FIG. 1. **Elution profile from Mono-Q column and SDS-PAGE of bovine brain PFK-2/FBPase-2.** PFK-2/FBPase-2 from bovine brain was reapplied into a Mono-Q column as described under "Results." The enzyme was eluted between 19 and 21 min. A fraction of the peak was subjected to gel electrophoresis in an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

TABLE I

*Purification of PFK-2/FBPase-2 from bovine brain*

| Steps | Protein | PFK-2 activity | Esp. Act. | Purification | Yield |
|---|---|---|---|---|---|
| | *mg* | *mU* | *mU/mg* | *-fold* | *%* |
| Extract | 25002 | 147 | 0.006 | 1 | 100 |
| 6–21% PEG | 4242 | 82.4 | 0.019 | 3.2 | 56 |
| Blue Sepharose | 3.44 | 48.4 | 14.1 | 2345 | 32.9 |
| Mono-Q | 0.71 | 31.6 | 44.5 | 7417 | 21.5 |
| Mono-Q reapplication | 0.15 | 13.9 | 92.7 | 15450 | 9.5 |

protein for the kinase and 29 milliunits/mg of protein for the bisphosphatase.

*Molecular Weights*—The $M_r$ 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-$^{32}$P]$P_2$ (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-$^{32}$P]$P_2$, 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 isoforms (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-$P_2$ (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-$P_2$ to 50% without changing the $V_{max}$ 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.

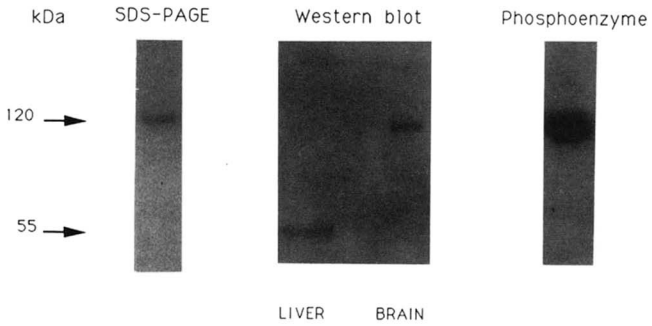


FIG. 2. **SDS-PAGE, Western blot, and fructose 2,6-bisphosphate labeling of brain PFK-2/FBPase-2.** The *left panel* is the Coomassie Blue stain of purified brain PFK-2/FBPase-2. The *center panel* is the immunoblot of brain and liver enzymes that were performed as described under "Experimental Procedures." The *right panel* shows SDS-PAGE electrophoresis of $^{32}$P-labeled brain enzyme with Fru-2,6-[2-$^{32}$P]$P_2$.

TABLE II

*Kinetic constants of bovine brain PFK-2/FBPase-2*

PFK-2 activity was determined as described under "Experimental Procedures" except that ATP concentration was kept at 5 mM and Fru-6-P was varied for the determination of $K_m$ for Fru-6-P; Fru-6-P was kept at 5 mM and ATP varied for the determination of $K_m$ for ATP; ATP was kept at 5 mM and Fru-6-P and citrate were varied for the determination of $K_i$ for citrate. FBPase-2 activity was determined as described under "Experimental Procedures" except that Fru-2,6-$P_2$ concentration was varied for the determination of the $K_m$; Fru-2,6-$P_2$ and Fru-6-P concentrations were varied, and NADP, phosphoglucose isomerase, and glucose-6-phosphate dehydrogenase were omitted for the determination of $K_i$ for Fru-6-P; Fru-2,6-$P_2$ and citrate were varied for the determination of $K_a$ for citrate.

| | $V_{max}$ | $K_m$ ATP | $K_m$ Fru-6-P | $K_i$ citrate |
|---|---|---|---|---|
| PFK-2 | 90 mU/mg | 55 μM | 27 μM | 35 μM |
| | $V_{max}$ | $K_m$ Fru-2,6-$P_2$ | $K_i$ Fru-6-P | $K_a$ citrate |
| FBPase-2 | 29 mU/mg | 70 μM | 20 μM | 16 μM |

*Phosphorylation of Brain PFK-2/FBPase-2 by Cyclic AMP-dependent Protein Kinase and Protein Kinase C*—To analyze further 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 of 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 $V_{max}$ 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

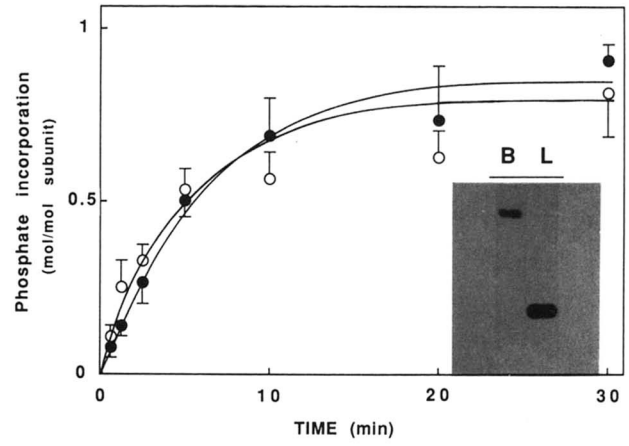


FIG. 3. **Phosphorylation of bovine brain and rat liver PFK-2/FBPase-2 by cyclic AMP-dependent protein kinase.** PFK-2/FBPase-2 from (*B*) brain (0.5 μM) and (*L*) liver (1 μM) were incubated in the presence of 2 milliunits/ml catalytic subunit of cyclic AMP-dependent protein kinase as described under "Experimental Procedures." At the indicated times, aliquots (40 μl) were removed, and the amount of [$^{32}$P]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 autoradiographied.

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 $NH_2$ terminus of the protein was blocked to Edman degradation. PFK-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 Gln-Val-Asn-Gln-His-Leu-Lys-Gly-Glu-Tyr. This sequence has no significant homology to any of the known sequences of other mammalian PFK-2/FBPase-2s (13, 15, 17, 33–34).

## DISCUSSION

We report here a procedure for the isolation of PFK-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 $M_r$ 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, 54, 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 milliunits/mg of protein for the bisphosphatase. No other significant brain forms of PFK-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 PFK-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 12 successive exons which are similar to those of the liver/skeletal muscle gene, but the heart gene has different exons at ends of the gene.The novel form expressed in brain has kinetic constants for substrates and allosteric effectors which are similar to those of the other bifunctional enzymes, particularly those of the heart form (10, 31). It shares common basic properties of all mammalian PFK-2/FBPase-2 such as homodimeric structure and bifunctionality. It forms a phospholabeled intermediate when incubated with Fru-2,6-[2-$^{32}$P] $P_2$, which suggests a common catalytic mechanism for the bisphosphatase reaction (3). It also shares some common antigenic determinants since it cross-reacts with antisera raised against the liver isozyme. In addition, total RNA from brain strongly hybridized with a near-full-length cDNA probe from liver, giving a major band of 6.8 kb and a minor one of 4 kb (23). All these properties suggest that it is a closely related structure to the other PFK-2/FBPase-2 isozymes. However, protein sequencing from a CNBr-derived peptide revealed no sequence homology to any other known PFK-2/FBPase-2. This lack of homology is not due to species differences since the bovine liver and bovine heart sequences have recently become available (14, 17), and they show no amino acid identity to the bovine brain CNBr sequence. In addition, the subunit molecular weight also is about twice that of all other mammalian PFK-2/FBPase-2s, including the form recently isolated from testis which has a molecular mass of 55 kDa and whose mRNA is 2 kb in size (13). A bifunctional enzyme from yeast, with a subunit molecular mass (93 kDa) of about twice that of mammalian forms, was recently cloned (36). This yeast enzyme has an analogous kinase/bisphosphatase domain structure to that found in the mammalian forms, but also has an additional extensive $NH_2$- and COOH-terminal sequence which has no homology to the mammalian forms. In addition, the active site phosphoacceptor (His-258 in the rat liver form) is a serine in the yeast enzyme, and this form is devoid of FBPase-2 activity (37). Recently, we have screened a bovine brain cDNA library with a human heart cDNA probe. A clone was isolated whose sequence revealed that the brain isozyme is encoded by a separate gene, which is different from those that encode the liver/skeletal muscle, heart, and testis forms, and which is specifically expressed in neural tissues.[2] 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.

The concentration of Fru-2,6-$P_2$ in mammalian tissues is controlled by the relative activities of PFK-2 and FBPase-2. The kinase/bisphosphatase activity ratio is close to 3 for the brain enzyme when both activities are determined under $V_{max}$ conditions. However, the bisphosphatase has a very high $K_m$ 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 (5 nmol/g) despite the low enzyme concentration (18, 21–23). In addition, the bisphosphatase would be expected to be inhibited *in vivo* by the concentrations of Fru-6-P normally found in brain (20 μM). Since Fru-6-P is also a substrate for the kinase and its cellular concentrations are close to the $K_m$ value for PFK-2, it is reasonable to postulate that the steady state Fru-6-P level controls brain Fru-2,6-$P_2$ concentration. An increase of Fru-6-P would result in a parallel increase of Fru-2,6-$P_2$ by virtue of increased kinase activity as well as decreased bisphosphatase activity via noncompetitive product inhibitor (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 ischaemia or hypoglycemia (21–23). These considerations do not apply to the other substrate of the kinase activity, since the $K_m$ 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 glycerol 3-phosphate or phospho(enol)pyruvate which act as allosteric effectors on liver PFK-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 $K_m$ for its substrate (2-fold activation at 5 μM Fru-2,6-$P_2$). 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 PFK-1 (39) and PFK-2 (present paper). When available, ketone body metabolism in brain increases citrate levels which

[2] 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-$P_2$ 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, while 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 $NH_2$- 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-$P_2$ 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. Dalmau, R. El-Maghrabi, and A. Lange for their help and for many valuable suggestions during the course of this work. The skillful technical assistance of C. Ortuño and T. Fisher is also acknowledged.

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