A New Transient Activator of Phosphofructokinase during Initiation of Rapid Glycolysis in Brain*

Susumu Ogushi, J. W. Randy Lawson, Geoffrey P. Dobson, Richard L. Veech, and Kosaku Uyeda

From the Department of Veterans Affairs Medical Center, Dallas, Texas 75216, the Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75223, and the National Institute of Alcohol Abuse and Alcoholism, Rockville, Maryland 20852

The tissue contents of previously known allosteric effectors of brain phosphofructokinase (EC 2.7.1.11) (PFK) and the kinetic behavior of isolated PFK were investigated during the initiation of rapid glycolytic flux in freeze-blown rat brain. Comparing 0- with 5-s brains revealed that there was a 4-fold drop in total tissue content of Fru-6-P and a 5.6-fold increase in Fru-1,6-Pz consistent with activation of PFK. Additionally, analysis of brain content showed a 15-fold increase in AMP, a 3-fold decrease in ATP, a 3-fold decrease in Pi, and a 1.6-fold increase in NH4+. There was no change in Fru-2,6-Pz, H+, citrate, or G国内外-1,6-Pz or the kinetic profiles of isolated PFK for ATP inhibition or Fru-2,6-Pz activation. We concluded that the observed change in PFK activity could be accounted for only partially by changes in the concentrations of adenine nucleotides and other known effectors.

High performance liquid chromatography fractions of extracts obtained from 5-s brains showed the activator with a mobility identical to ribose 1,5-Pz and gave 2 nmol/g (wet weight) at 0 s, 10 nmol/g at 5 s, and 2 nmol/g at 20 s. Assay of PFK in the presence of effectors determined to be in tissue at 5 s showed that addition of 10 nmol/ml ribose 1,5-Pz gave a 4-fold activation of PFK. Based on the rapidity of its formation, its potency of activation, and its similarity in chemical properties to authentic ribose 1,5-Pz, we conclude that ribose 1,5-Pz served as the initial activator of PFK in brain.

The classic studies that established PFK as a control point in glycolysis compared the metabolite contents of brains of adult mice, dropped whole into Freon, with those found in heads decapitated for various time periods prior to their immersion into Freon. In the first few seconds following decapitation before dropping the heads into Freon the studies calculated that there was 4-7-fold increase in glycolytic rate and found that the contents of glucose, Glu-6-P, and Fru-6-P decreased while all metabolites from Fru-1,6-Pz to lactate increased. These findings were interpreted as representing activation of phosphofructokinase (PFK) caused by increases in the positive allosteric effectors ADP, AMP, Pi, and Fru-1,6-Pz, and by a slight decrease (5%) in ATP, which is a potent inhibitor (2). The inhibition of PFK by ATP was thought to be overcome by small changes in any one of these activators, and simultaneous changes have been considered to be particularly effective (2, 3). However, this interpretation may not be consistent with the known actions of these effectors on PFK activity in vitro where physiological concentrations of ADP, AMP, Pi, and Fru-1,6-Pz are present either alone or together. Later the strong ATP inhibition was found to be overcome by Fru-2,6-Pz, the most potent activator of PFK. Although Fru-2,6-Pz is known to occur in brain as well as other tissues (4, 5), its role in the regulation of PFK activity in brain has not been clearly defined. Painwels and Trouet (6) showed that the Fru-2,6-Pz content in various types of cultured brain cells did not change at 30 min after the induction of respiratory chain inhibition. As noted above, the most significant changes in glycolysis probably occur within the first few seconds of ischemia. Consequently, it is important to determine the metabolic changes immediately after the rapid activation of glycolysis in brain in order to determine the factor(s) responsible for triggering the activation of PFK and glycolysis. For this purpose we have used the rapid freeze-blowing technique to obtain brain samples in which glycolysis was activated severalfold in less than 5 s and analyzed changes in the key metabolites of PFK.

EXPERIMENTAL PROCEDURES

Materials—[14C]Adenine was purchased from Du Pont-New England Nuclear. Rib-1,5-Pz was synthesized from Rib-5-P by the phosphoglucomutase reaction (EC 5.4.2.2) in the presence of Glu-1,6-Pz (7), and glucose-6-P dehydrogenase was added to drive the reaction to completion (8). The product was purified by chromatography on a Dowex 1-formate or HCO3 column (7). Rabbit muscle PFK was purified as described (9, 10). Phosphobibosylpyrophosphate synthetase was purchased from Sigma. Pure phosphoribosylpyrophosphate synthetase was kindly provided by Dr. Robert L. Switzer (University of Illinois, Urbana). Adenine phosphoribosyltransferase was prepared according to the procedure of Hershey and Taylor (11). An Aminex HPX-87C column was purchased from Bio-Rad, and a CarboPac PA1 column was purchased from Dionex Corp. (Sunnyvale, CA). All other enzymes were obtained from either Boehringer Mannheim or Sigma. All other chemicals and reagents were analytical reagent grade and purchased from commercial sources.

Rats—Male Wistar rats weighing 200-250 g from Charles River Breeding Laboratories (Wilmington, MA) were used. All were fed ad libitum with the standard NIH rat diet.

Removal of Brain—The supratentorial portion of the brain was rapidly expelled by a jet of air into a hollow disc using the brain-blowing device (12). For zero time controls, the disc was precooled to

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*This work was supported by a grant from the Department of Veterans Affairs Medical Center and by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK16184. 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.

† To whom reprint requests should be addressed: VA Medical Center, 4500 S. Lancaster Rd. Dallas, TX 75216. Tel.: 214-372-7028.
Brain Phosphofructokinase Activator

liquid nitrogen temperature, resulting in freezing of the wafer-thin (1-mm) tissue in less than 1 s. For stimulated glycolysis, the brain was blown into the disc at room temperature for predetermined time intervals and then plunged into liquid nitrogen for quick freezing.

Metabolic Measurements—Fru-2,6-P₂ was extracted with an alkaline buffer (13) and analyzed by the method of Thomas and Uyeda (14). A sample (300 mg) of frozen brain powder was deproteinized at 80°C for 10 min with 0.9 ml of buffer containing 50 mM Tris/HCl, pH 8.0, 0.1 mM EDTA, and 10 mM NaF. After homogenization and centrifugation, the clear supernatant solution was diluted 1:4 with 0.02 M NH₄OH and adsorbed on Dowex 1-chloride columns (0.5 x 2.0 cm) that had been equilibrated with 0.02 M NH₄OH and assayed. All other metabolites were determined fluorometrically or spectrophotometrically on neutralized 36% perchloric acid extracts using enzymatic techniques described previously (12, 15, 16). Total tissue HCO₃⁻ content was determined and used to estimate intracellular pH as described by King et al. (17). Free cytosolic ADP, AMP, and Fru-1,6-P₂ were calculated as described previously (18, 19). For the free ADP and AMP calculations, the relevant equilibrium and acid dissociation constants were adjusted for the intracellular pH estimated in these experiments as detailed earlier (20). An intracellular free (Mg²⁺) concentration of 1.0 mM was assumed for these calculations (21, 22).

Purification of PFK from Rat Brain—PFK was isolated from rat brain by one-step affinity chromatography on ATP-Sepharose (10) as described previously for rat heart (23) except that the polyethylene glycol fractionation steps were omitted. Briefly, 1 g of frozen brain powder was suspended in 5.0 ml of ice-cold extraction buffer containing 50 mM Tris/phosphate buffer, pH 8.0, 10 mM dithiothreitol, 100 mM KF, 1 mM benzamidine, 0.5 mM phenylmethylsulfonil fluoride, 0.2 mM EDTA, and 2 mM EGTA and homogenized at 4°C using a Polytron homogenizer (Brinkmann Instruments). After centrifugation at 30,000 x g for 30 min at 4°C, the clear supernatant solution was applied to an ATP-Sepharose column (0.5 x 2 cm) and washed first with 10 ml of cold extraction buffer followed by 5 ml of the buffer mixture containing 0.05 M AMP over 15-20 min. PFK was eluted with 5 ml of extraction buffer containing 0.2 mM each ADP, ATP, Fru-1,6-P₂, and aldolase (0.4 unit). This procedure recovered 90% of the PFK activity in the original supernatant solution (15 units/g, wet weight) with a specific activity of 50-60 units/mg of protein. PFK was concentrated immediately after elution using the Centrifloc method (Amicon) and used in the assays or stored at −90°C until use within 5 days.

Assay Methods for PFK—The activity of PFK was assayed at optimum assay conditions. The assay mixture contained in a final volume of 1 ml 50 mM Tris/HCl, pH 8.0, 0.1 mM EDTA, 6 mM MgCl₂, 4 mM ammonium sulfate, 2.5 mM dithiothreitol, 0.16 mM NADH, 1 mM each Fru-6-P and ATP, and aldolase (0.4 unit). The reaction was initiated by the addition of PFK, and the decrease in absorbance at 340 nm was measured at 25°C. One unit is defined as the amount of enzyme which catalyzes the formation of 1 µmol of fructose 1,6-P₂ over 1 min.

Allosteric kinetic properties of PFK were determined in 1 ml of reaction mixture containing 50 mM Hepes, pH 7.25, 0.2 mM EDTA, 0 mM MgCl₂, 1 mM NH₄Cl, 0.16 mM NADH, 2.5 mM dithiothreitol, indicated amounts of Fru-6-P and ATP, and desalted aldolase (0.4 unit), glyceraldehyde 3-phosphate dehydrogenase (0.4 unit), triose-P isomerase (2.4 unit), and rabbit muscle PFK (15 milliunits). Activity is expressed as v/Vₘₚ₉, where v is the activity under conditions for determination of allosteric kinetic PFK, and Vₘₚ₉ (maximum activity) is the activity determined in the same reaction mixture except 1 unit Fru-6-P and 0.4 mM ATP were used.

Assay Methods for Rib-1,5-P₂ and the Activator—Rib-1,5-P₂ was assayed after converting it to Rib-5-P by acid hydrolysis in 0.03 N HCl for 60 min at 37°C. This acid hydrolysis treatment resulted in quantitative conversion to Rib-5-P, and Rib-5-P was assayed by the coupled reactions catalyzed by phosphoribosylpyrophosphate synthetase and adenosine phosphoribosyltransferase as described (24). We found that it was necessary to remove interfering substances in brain extracts before this assay could be performed. Thus, sugar bisphosphate in the charcoal-treated brain extract was adsorbed on a Dowex 1-HCO₃⁻ column (1 x 3 cm), washed with 25 ml of 0.15 M NaHCO₃ or NH₄HCO₃, which elutes sugar phosphate, and sugar bisphosphate was eluted with 25 ml of 0.25 M NaHCO₃ or NH₄HCO₃. The activator and Rib-1,5-P₂ also were assayed based on their ability to activate PFK as follows. The reaction mixture was the same as the allosteric kinetic assay except 1 mM Fru-6-P, 10-50 pmol of Rib-1,5-P₂, and 15 milliunits of PFK were added and incubated for 2 min. The reaction was initiated with addition of 1.8 mM ATP. The activation of PFK was directly proportional to Rib-1,5-P₂ concentration in the range of 10-60 µmol under these conditions (Fig. 1). The specific activity of Rib-1,5-P₂ was 84 units/µmol, where 1 unit is defined as the amount of pentose-P₂ which increases PFK activity by 1 unit under these conditions.

RESULTS

Evidence for Increased Glycolysis—The lactate content of brain increased from 2.1 ± 0.5 to 2.8 ± 0.8 µmol/g at 5 s, an increase of 0.7 µmol/g (Fig. 2A), which corresponds to a production rate of 8.4 µmol/g/min. Over the same time period, total Fru-1,6-P₂ increased from 0.03 to 0.17 µmol/g for a rate of production of 2.8 µmol/g/min (Fig. 2B). This corresponds to 5.6 µmol of lactate per g/min, which is in good agreement with the measured rate of production of lactate (1, 25-27). Thus, glycolysis was accelerated at least 7-fold within 5 s of the onset of the induced glycolysis, which agrees well with the estimation in the ischemic brain samples reported by Lowry et al. (1). Over the next 2 min the rate of glycolysis progressively slowed. The rate of lactate production decreased to 3.6 µmol/g/min from 5-10 s and then further to 1.0 ± 0.4 µmol/g/min over the next 110 s.

Metabolic Changes during the Activation of Glycolysis—Within the first 5 s, Fru-6-P fell to a nadir of 25% of the control value while total Fru-1,6-P₂ increased almost 6-fold (Fig. 2B). Over the next 115 s, Fru-6-P and total Fru-1,6-P₂ accumulated, with Fru-6-P rising to 125% and Fru-1,6-P₂ to 1600% of control values after 2 min. In contrast, Fru-2,6-P₂ decreased slightly in 10 s and slowly increased thereafter. By 5 s, ATP decreased to 35% of the control level and by 20 s, to about 15-20% of control where it remained over the next 100 s (Fig. 2C). In contrast, total AMP increased 25-fold after 10 s of ischemia and remained constant thereafter. Total ADP increased 30% by 10 s and then returned to control values by 20 s without further change over the next 100 s. Based on these metabolic data the most significant activation of glycolysis and PFK occurred within 5 s. Therefore, the concentrations of these and the other reported effectors of PFK activity were assessed at that time and are presented.

FIG. 1. The standard curve for PFK activity versus Rib-1,5-P₂. Indicated concentrations of Rib-1,5-P₂ were assayed using the allosteric kinetic PFK assay as described under "Experimental Procedures."
tissue was processed for metabolite measurements. The results are device and either frozen immediately in liquid nitrogen for control the total (t) and calculated free (f) concentrations of ADP, AMP, and citrate are from Fig. 2. (*) indicates significance of the p < 0.001 pH calculation as described under “Experimental Procedures.” Both Fru-1,6-Ps are reported. The values for ATP, Fru-2,6-P, Fru-1,6-Pn, level usine the unaaired Student’s t test (two-tailed).

FIG. 2. Effect of rapid activation of glycolysis on metabolite contents in rat brain. Brains were obtained using a brain-blowing device and either frozen immediately in liquid nitrogen for control values (0 s) or maintained at 25 °C for predetermined times up to 2 min prior to quick freezing in liquid nitrogen. After freezing, the tissue was processed for metabolite measurements. The results are mean values (±S.E.) for three to five brains. For more details, see “Experimental Procedures.”

TABLE I

| Metabolite, ratio, or pH | Before ischemia | After ischemia |
|-------------------------|----------------|---------------|
| ATP                     | 2,600          | 900*          |
| Fru-6-P                 | 40             | 10*           |
| Fru-1,6-P<sub>t</sub>   | 0.1            | 3.1*          |
| Fru-1,6-P<sub>f</sub>   | 30             | 170*          |
| AMP (t)                 | 0.0            | 10*           |
| AMP (f)                 | 60             | 1,000*        |
| P<sub>i</sub>           | 2,300          | 7,800*        |
| NH<sub>4</sub>          | 270            | 420*          |
| Fru-2,6-P               | 2.6            | 2.3           |
| Glc-1,6-P<sub>t</sub>  | 57             | 65            |
| Citrate                 | 325            | 268*          |
| [HCO<sub>3</sub>]<sup>-</sup> | 16,850       | 17,120        |
| pH                      | 7.3            | 7.3           |
| [Fru-1,6-P<sub>t</sub> (f) [ADP (f)] | 0.04 | 3.0 |
| [Fru-6-P] [ATP] | [ADP (f)] [P]<sup>i</sup> | 30,600 | 1,300 |

in Table I. After 5 s the calculated free Fru-1,6-P<sub>f</sub> increased 30-fold from 0.11 to 3.9 nmol/g. Similarly, the free AMP increased 16-fold from 0.61 to 9.7 nmol/g whereas the free ADP increased only 2-fold from 37 to 88 nmol/g. Of note, the hexose bisphosphates Fru-2,6-P<sub>t</sub> (2.6–2.3 nmol/g) and Glc-1,6-P<sub>t</sub> (57–65 nmol/g) did not change significantly, in contrast to Fru-1,6-P<sub>t</sub>, inorganic phosphate (P<sub>i</sub>) increased 3.4-fold, and NH<sub>4</sub> increased 1.6-fold after 5 s of ischemia. Citrate decreased slightly (20%) but significantly at this time. The calculated intracellular pH did not change over this brief time period.

The cytosolic phosphorylation potential calculated from the metabolites of the combined glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, and lactate dehydrogenase reactions (19) dropped 25-fold, and the mass action ratio for PFK, also calculated using the free metabolite values, increased 75-fold, consistent with the marked activation of PFK.

Effect of Metabolite Changes on PFK Activity in Vitro—In order to delineate further the regulatory roles of these metabolites and effectors and to investigate the possibility of a covalent modification of PFK, detailed kinetic analyses were done using PFK isolated from 0-s control and 3-s stimulated brains. These in vitro kinetic studies were done with varying concentrations of ATP, AMP, Fru-2,6-P<sub>t</sub>, and Fru-6-P in the presence of other metabolites at the concentrations and pH values determined above (Table I). To eliminate uncertainties as to whether total or free AMP or Fru-1,6-P<sub>2</sub> content was appropriate (2,19,26), a range of concentrations that included the free and total amounts for these metabolites was used.

The in vitro kinetic studies demonstrated that in spite of large increases in the activators, AMP, P<sub>i</sub>, Fru-1,6-P<sub>2</sub>, the constant presence of Fru-2,6-P<sub>t</sub> and a decrease in the inhibitors ATP and citrate, we could not account for the activation of PFK under the conditions comparable to those in the brain during rapid activation of glycolysis (Table II). This is because the concentration of Fru-6-P decreased from 40 μM (in the control) to 10 μM in 5 s, and thus drop off the 3-4-fold activation of PFK provided by the changes in ATP and AMP. This inability to explain adequately the rapid activation of PFK seen in 5-s samples prompted us to search for a yet unidentified activator.

Brain PFK Activator—Preliminary experiments demonstrated that rat brain contained an activator of PFK in addition to Fru-2,6-P<sub>t</sub>. The activator was found to be acid labile, and thus its concentrations presented in Fig. 3 (solid circles) were determined by the PFK activation assay before and after the acid treatment as described under "Experimental Procedures." The acid treatment specifically hydrolyzed this activator. After this activator was identified as Rib-1,5-P<sub>t</sub>, described below, Rib-1,5-P<sub>t</sub> in these samples (Fig. 3, open circles) was assayed for Rib-5-P<sub>t</sub> by the coupled enzymatic assay after the acid treatment. As shown in the figure, the values for the activator and Rib-1,5-P<sub>t</sub> agree well. The concentration of this activator varied rapidly with time after onset of glycolysis activation. Within 2 s of initiating increased glycolysis, the concentration of the activator increased.

TABLE II

| PFK activity in vitro | Control 5-s ischemic |
|-----------------------|----------------------|
| \( u/V_{max} (\text{mg} \text{ min}^{-1}) \) | \( u/V_{max} (\text{mg} \text{ min}^{-1}) \) |
| AMP (t)/Fru-1,6-P<sub>t</sub> (t) | 0.20 (1.8) |
| AMP (t)/Fru-1,6-P<sub>t</sub> (f) | 0.21 (1.9) |
| AMP (t)/Fru-1,6-P<sub>t</sub> (t) | 0.66 (5.9) |
| AMP (t)/Fru-1,6-P<sub>t</sub> (f) | 0.64 (5.8) |
acid containing 1 were prepared as described under "Results." Each sample was assayed the acid treatment as described under "Experimental Procedures." The charcoal-treated extract was adsorbed on a CarboPac to the rapid decrease in phosphatase. Its ability to activate PFK was lost completely after 17.6 min and was desalted by passing through a Bio-Gel purified by HPLC anion exchange chromatography as follows.

The results were mean values (+S.E.) for 8-10 brains.

approximately 2.5-fold and 6-fold at 5 s. Thereafter its concentration rapidly decreased to the basal level by 20 s. This rapid increase in activator concentration corresponded closely to the rapid decrease in Fru-6-P and the simultaneous increase in Fru-1,6-P2 reported previously (Fig. 2B), indicating immediate activation of PFK. These results suggested that this activator may be responsible for the initial activation of PFK in the brains.

Isolation of a PFK Activator—Tissue extract for the isolation of the PFK activator was prepared by the procedure that was developed for the isolation and determination of phosphoribosylpyrophosphate. Briefly, powdered freeze-clamped ischemic brain (1-2 g) was suspended in 2 volumes of methanol at -30 °C and homogenized in cold 0.55 mM perchloric acid containing 1 mM EDTA. Fru-2,6-P2 was completely destroyed by the perchloric acid treatment, but the recovery of the PFK activator was over 95%. The mixture was quickly centrifuged, and the supernatant solution was neutralized with cold 3 M KHCO3. An equal volume of acid-washed charcoal (40 mg/ml) was added, and the charcoal was removed by centrifugation followed with filtration. The activator was purified by HPLC anion exchange chromatography as follows. The charcoal-treated extract was adsorbed on a Bio-Pac PA1 column. It was eluted at 17.50 min, which was identical (Fig. 4B). The half-lives of the isolated activator and the activator isolated from brain after 5 s of the stimulated glycolysis were identical (Fig. 4A). Furthermore, the rate of Rib-1,5-P2 disappearance and the rate of Rib-5-P formation by the acid treatment were also identical (Fig. 3B). The half-lives of the isolated activator and the activator isolated from brain after 5 s of the stimulated glycolysis were identical (Fig. 4A). Furthermore, the rate of Rib-1,5-P2 disappearance and the rate of Rib-5-P formation by the acid treatment were also identical (Fig. 4B). The half-lives of the isolated activator and the activator isolated from brain after 5 s of the stimulated glycolysis were identical (Fig. 4A).

Characterization of the Activator—The activator was not adsorbed by charcoal. However, it was adsorbed on Dowex 1-formate and eluted with the same concentration of formate as Glc-1,6-P2 and Fru-1,6-P2, suggesting that it was a bisphosphate. There was no other activator eluted with other formate concentrations known to elute monophosphates or triphosphates. The activator was purified further by HPLC on Partisil 10 SAX (Whatman, OR) and treated with alkaline phosphatase. Its ability to activate PFK was lost completely by the phosphatase treatment. When the alkaline phosphatase-treated sample was chromatographed by HPLC on an Aminex HPX-87C column (Bio-Rad), a major peak of sugar detected corresponded to ribose, which was eluted at 21.18 min. All the other sugars examined including glucose, galactose, mannose, fructose, arabinose, and tagatose were eluted before 16.5 min. A trace (<5%) of glucose was also detected. The activator was partially purified from ischemic brain as described under "Experimental Procedures." Flow rate = 1 ml/min. The sugar bisphosphates were detected with a pulsed amperometric detector (Dionex).

- Sugar bisphosphate
- Elution time
- 6-P-glucose
- 11.34 min
- Glc-1,6-P2
- 16.78 min
- Ribulose-1,5-P2
- 1.78 min
- Fru-2,6-P2
- 33.98 min
- Fru-1,6-P2
- 67.71 min
- Sedoheptulose 1,7-P2
- 101.09 min
- Rib-1,5-P2
- 77.99 min
- PFK activator
- 17.56 min

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- PFK activator
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TABLE III

| Purification step | Total activity | Amount of Rib-1,5-P2 | Yield % |
|------------------|----------------|---------------------|---------|
| Extract          | 773            | 9.30                | 100     |
| Charcoal treatment | 734           | 8.74                | 85      |
| Gel filtration (Bio-Gel P-2) | 657       | 7.82                | 85      |
| HPLC (CarboPac PA1) | 355        | 4.23                | 46      |
| Gel filtration (Bio-Gel P-2) | 317       | 3.77                | 41      |

TABLE IV

HPLC chromatography of sugar bisphosphate on CarboPac PA1 column

Sugar bisphosphate (10-20 nmol) was loaded on the CarboPac PA1 column and eluted with 0.15 N NaOH, 0.3 M sodium acetate at 25 °C. The PFK activator was partially purified from ischemic brain as described under "Experimental Procedures." Flow rate = 1 ml/min. The sugar bisphosphates were detected with a pulsed amperometric detector (Dionex).

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confirming that the PFK activator was Rib-1,5-P₂.

With time were the same as that of the activator, thus results show (Fig. 3, open circles) that the changes in Rib-1,5-P₂ and brain PFK activator. The reaction mixture contained in 1.0 ml of 0.03 N HCl, synthetic Rib-1,5-P₂ (1.4 nmol) or the activator (1.4 nmol), which was purified with a Carbopac column as described under "Experimental Procedures." B, the same reaction mixture (1 ml) for each time point in 1.0 ml of 0.03 N HCl, synthetic Rib-1,5-P₂ (1.4 nmol) or the activator (1.4 nmol) as described under "Experimental Procedures." For the same reaction mixture (1 ml) for each time point was also assayed for Rib-5-P using the coupled enzyme assay for Rib-5-P as described under "Experimental Procedures."

**Fig. 4. Comparison of acid hydrolysis rate of synthetic Rib-1,5-P₂ and brain PFK activator.** The reaction mixture contained in 1.0 ml of 0.03 N HCl, synthetic Rib-1,5-P₂ (1.4 nmol) or the activator (1.4 nmol), which was purified with a Carbopac column as described under "Results." A, at indicated time intervals an aliquot (100 µl) was removed, neutralized to pH 8.0, and assayed for Rib-1,5-P₂ using the PFK activation assay as described under "Experimental Procedures." B, the same reaction mixture (1 ml) for each time point was also assayed for Rib-5-P using the coupled enzyme assay for Rib-5-P as described under "Experimental Procedures."

**Fig. 5. Effect of Rib-1,5-P₂ on brain PFK.** The reaction mixture in 1.0 ml contained 50 mM Heps, pH 7.25, 50 mM KCl, 10 mM MgCl₂, 40 mM EDTA, 0.18 mM NADH, 0.2 mM phosphoenolpyruvate, pyruvate kinase (1 unit), lactate dehydrogenase (1 unit), 5 mM phosphate, 0.35 mM NADH, 60 µM Glic-1,6-P₃, 0.3 mM citrate, 5 µM Fru-1,6-P₂, 0.9 mM ATP, indicated concentration of AMP, 10 µM Fru-2,6-P₂, 1.2 µM Fru-1,6-P₂, and varying concentrations of synthetic Rib-1,5-P₂. Brain PFK (15 milliuunits) was added to initiate the reaction.

results show (Fig. 3, open circles) that the changes in Rib-1,5-P₂ with time were the same as that of the activator, thus confirming that the PFK activator was Rib-1,5-P₂.

**Activation of Brain PFK by Rib-1,5-P₂.** When the activation of brain PFK by Rib-1,5-P₂ was examined under the conditions similar to the 5-s brain, the Kₐₛ values were approximately 4 and 7 µM depending on the presence of 1 or 10 µM AMP, respectively (Fig. 5). Interestingly, these observed Kₐₛ values were near the physiological concentration range of Rib-1,5-P₂ found in the zero time (2 nmol/g) and 5-s sample (10 nmol/g). The results also showed strong synergistic activation of the enzyme by Rib-1,5-P₂ and AMP.

**DISCUSSION**

Brain removed by brain blower into a room temperature disc caused a rapid acceleration of glycolysis, with maximal activation occurring during the first 5 s. This activation was associated with significant increases in AMP, Fru-1,6-P₂, NH₃, and P; and decreases in ATP, citrate, and Fru-6-P content. Based on the kinetic analyses of PFK, the changes in ATP and AMP were the most important in contributing to the initial increase in PFK activity and rate of glycolysis. Although PFK was activated by these changes in ATP and AMP, the activation was offset by a dramatic decline in Fru-6-P concentration. The adenine nucleotide changes acted to maintain PFK activity near control level. Even though its concentration did not change, Fru-2,6-P₂ was required for optimizing PFK activity under both control and the stimulated glycolysis conditions. During the initial 5 s, none of the other well known effectors of PFK activity appeared to be important, namely pH, NH₃, citrate, Fru-1,6-P₂, or Glu-1,6-P₃, either alone or when present together. No kinetic evidence for a stable covalent modification of PFK was observed.

The most important contribution of this work was the discovery of a new activator of PFK in brain under the conditions in which glycolysis was rapidly activated. The activator was formed within 2-5 s after the initiation of the activation, and the time course of its formation corresponded exactly with the decrease in Fru-6-P and the concomitant increase in Fru-1,6-P₂, consistent with activation of PFK and an increase in glycolysis (compare Fig. 2 with Fig. 3). There was at least a 5-fold increase in the activator concentration within 5 s whereas Fru-6-P decreased 7-fold and Fru-1,6-P₂ increased nearly 10-fold during the same period. Furthermore, the kinetic studies carried out with brain PFK in the presence of all the effectors indicated that the enzyme was activated by the same concentration range of Rib-1,5-P₂ (2-10 µM) found in the control and 5-s brains. Thus, the rapidity and the physiological range of its concentration strongly suggested the in vivo significance of this sugar bisphosphate in the activation of PFK in these brains.

There appeared to be no other activator present in those extracts of the brain samples prepared after 2-5 s of the stimulated glycolysis. Special care was taken in the preparation of the brain extract, employing the method originally developed for the preparation of phosphoribosylpyrophosphate, so that acid-labile activators such as phosphoribosylpyrophosphate and Rib-1,5-P₂ were not destroyed. Using this method the recovery of Rib-1,5-P₂, as determined by preparing an extract from control brain with a known amount of Rib-1,5-P₂ added, was over 95% whereas that of phosphoribosylpyrophosphate was about 80%. It is still possible that an extremely acid-labile activator such as Fru-2,6-P₂ would have been missed in this study. (In the above extract, Fru-2,6-P₂ had been intentionally destroyed with mild acid treatment in order to avoid complication in the assay for the new activator.) This possibility was ruled out, however, since the extract made in neutral or alkaline medium also contained mainly the same activator in the same amount as judged by HPLC and ion exchange chromatography. The observations that 1) no other activator was found in the eluate of HPLC and 2) the overall recovery of the activator during the Rib-1,5-P₂ isolation was reasonably high considering the instability of the sugar bisphosphate strongly suggested that Rib-1,5-P₂ was...
the key activator of the enzyme in these brains.

The chemical structure of this PFK activator isolated from brain is consistent with its identification as Rib-1,5-P₂ based on the following lines of evidence. 1) The ion exchange chromatography indicated that it is a sugar bisphosphate; 2) Its elution time from an HPLC ion exchange column was identical to that of synthetic Rib-1,5-P₂; 3) Upon acid hydrolysis followed by alkaline phosphatase treatment, it yielded ribose; 4) It contained an acid-labile phosphate, and the rate of acid hydrolysis was identical to that of Rib-1,5-P₂ and 5) Upon acid hydrolysis it yielded Rib-5-P in stoichiometric amounts. Although we cannot completely rule out another pentose 1,5-P₂ such as arabinose 1,5-P₂, it is unlikely for two reasons. 1) The assay for Rib-5-P using pure phosphoribosylpyrophosphate synthetase is specific for Rib-5-P, and arabinose 5-P would not serve as a substrate. The acid hydrolysate of the HPLC-purified activator yielded Rib-5-P in a stoichiometric amount. 2) Ribose, not arabinose, was detected by HPLC in the alkaline phosphatase-treated sample. Furthermore, Rib-2,5-P₂ and Rib-3,5-P₂ were ruled out because of their acid stability.

The apparent affinity of brain PFK for Rib-1,5-P₂ was approximately nine times weaker than Fru-2,6-P₂, which in turn was about eight times stronger than Glc-1,6-P₂. This difference in the activation by Rib-1,5-P₂ and Glc-1,6-P₂ was similar to the results reported using erythrocyte PFK (8).

It is now generally accepted that Fru-2,6-P₂ plays an important role in regulation of PFK activity in liver (28-30) and brain. In vivo the enzyme is strongly inhibited. Consequently, Fru-2,6-P₂, the product of the enzyme reaction, Fru-1,6-P₂, is able to maintain its activity in an autocatalytic manner, especially in the presence of AMP and Fru-2,6-P₂. Such an explanation is suggested by the plot shown in Fig. 6, comparing the time course of Rib-1,5-P₂ formation (from Fig. 3) with that of Fru-1,6-P₂ formation. The total Fru-1,6-P₂ concentration increased from 31 nmol/g in the control brain to 106 nmol/g in 5 s and continued to increase slowly beyond 10 s when Rib-1,5-P₂ dropped to near baseline. Kinetic studies performed with isolated PFK in vitro approximating the in vivo conditions, however, indicated that Fru-1,6-P₂ (higher than 150 μM) did not activate the enzyme but inhibited approximately 15%. Such an inhibition probably does not occur in vivo, however, because the PFK concentration in rat brain is approximately 2 μM and the Fru-2,6-P₂ concentration is 2.6 μM. In contrast, the PFK concentrations in these kinetic studies were 3 orders of magnitude less than that in vivo. Consequently, additional sugar bisphosphate could bind and activate the enzyme in vivo. Nevertheless, the above observations seem to support the idea that Rib-1,5-P₂ served as a trigger or an accelerator of PFK activation in the initial 5 s, and it is possible that once the enzyme has been activated it is no longer needed.

The demonstration, for the first time, of an in vivo role for Rib-1,5-P₂ in animal tissue raises several intriguing questions. First, the enzymes responsible for Rib-1,5-P₂ synthesis and degradation are not known. The observation that the turnover of Rib-1,5-P₂ in brain was extremely rapid suggests that the enzymes involved in its synthesis and degradation must be tightly regulated. Although Rib-1,5-P₂ can be synthesized in vitro from Rib-1-P and Glc-1,6-P₂ catalyzed by phosphoglucomutase (EC 5.4.2.2) (7), considerations of Kₐ₉ substrate levels and the equilibrium constant make this mechanism unlikely in vivo. Finally, levels of Rib-5-P increased from 10 to 20 μM in the 0- and 5-s samples, raising the possibility that this metabolite of the hexose monophosphate pathway may be the substrate for Rib-1,5-P₂ production. This possibility would provide a mechanistic explanation for the coordinated control of the initial portion of the glycolytic pathway and the hexose monophosphate pathway (31,32) and the relationship of these pathways during the control of growth (33,34).

Acknowledgements—We wish to thank Dr. Robert L. Switzer (University of Illinois) for pure Escherichia coli phosphoribosylpyrophosphate synthase and Dr. Sarah McIntire for her critical reading of this manuscript.

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A new transient activator of phosphofructokinase during initiation of rapid glycolysis in brain.

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J. Biol. Chem. 1990, 265:10943-10949.

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