Unequivocal demonstration of fructose-1,6-bisphosphatase in mammalian brain*

(gluconeogenesis/purification/noncrossreactivity/antiserum/fructose-phosphate "futile cycle")

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ABSTRACT   Fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase; EC 3.1.3.11) has been found in rat brain and identified unequivocally. The enzyme has been purified to 95% homogeneity by standard procedures, including adsorption to a phosphocellulose column followed by elution with substrate. The purified enzyme exhibits a broad optimum above pH 7.6. Both fructose 1,6-bisphosphate and sedoheptulose 1,7-bisphosphate are substrates of this enzyme; the hydrolysis of the latter occurs at about 20% of the rate of the former, and the K_m for fructose 1,6-bisphosphate is approximately 1.32×10^{-4} M. 5'-AMP, an inhibitor of other mammalian-fructose-1,6-bisphosphatases, is without effect, and in further contrast with the other enzymes there is no metal requirement for activity. Purified brain enzyme fails to crossreact with the antibody prepared against the purified liver fructose-1,6-bisphosphatase. On the other hand, antiserum produced against the brain fructose-1,6-bisphosphatase quantitatively precipitates the enzyme activity and forms precipitin bands with preparations of brain fructose-1,6-bisphosphatase.

Fructose-1,6-bisphosphatase (Fru-P_2ase; D-fructose-1,6-bisphosphate 1-phosphohydrolase; EC 3.1.3.11), one of the key gluconeogenic enzymes, is found in liver, kidney, muscle, and plants and has been studied extensively (for reviews see refs. 1 and 2). In surveying various tissues for the enzyme, Krebs and Woodford (3) were unable to demonstrate its presence in brain; Phillips and Coxon (4), however, presented preliminary evidence for the presence of the enzyme which can account for their observed incorporation of [^{14}C]pyruvate into glycogen in brain slices. Recently, in connection with a study of the inhibition of glycolysis by phospholipid in brain, we have demonstrated the conversion of [^{14}C]fructose 1,6-bisphosphate (Fru-P_2) into [^{14}C]fructose 6-phosphate (5), presumptive evidence for the existence of a phosphatase. In this paper we describe the isolation, purification, and characterization of a specific Fru-P_2ase from mammalian brain (6).

MATERIALS AND METHODS

Materials

Sodium salts of Fru-P_2, glucose 6-phosphate, fructose 6-phosphate, sedoheptulose 1,7-bisphosphate, glucose 1-phosphate, fructose 1-phosphate, β-glycerophosphate, 5'-AMP, and the tetracyclohexylammonium salt of glucose 1,6-bisphosphate were purchased from the Sigma Chemical Co. Tetracyclohexylammonium glycerol 1,3-bisphosphate was from Calbiochem. All other reagents were the best grade available.

Phosphocellulose P11, obtained from Whatman, Inc., was washed according to the procedure of Han *et al.* (7) and finally equilibrated with 10 mM acetate buffer, pH 5.7. Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Inc. Acrylamide, *N,N'*-methylene bisacrylamide, and *N,N,-N',N'*-tetramethyl ethylenediamine (TEMED) were from Bio-Rad Laboratories. Agar gel plates for Ouchterlony double immunodiffusion were obtained from Hyland.

Methods

The standard incubation mixture for assay of Fru-P_2ase contained 100 mM Tris·HCl buffer, pH 7.6/150 mM KCl/0.1 mM Fru-P_2 in a total volume of 1 ml. After incubation at 37° for 30 min, the reaction was terminated by the addition of 0.25 ml of 25% trichloroacetic acid. Precipitated protein, if any, was removed by centrifugation and the supernatant was assayed by the procedure of Ames (8) for inorganic phosphate (P_i) released from the substrate. Alternatively, Fru-P_2ase was assayed spectrophotometrically in the coupled enzyme system of Pontremoli *et al.* (9).

Isolation and purification of Fru-P_2ase from brain, by a procedure described by Traniello *et al.* (10) for purification of rabbit liver enzyme with minor modification, is outlined as follows. All operations were carried out at 0–4°.

Step I. Low-speed supernatant: Brain (12 g) from 12 male Osborne–Mendel rats (weighing 150–200 g) was homogenized with three volumes of 0.15 M KCl containing 0.01 M NaHCO$_3$ for 1–2 min and the homogenate was centrifuged at 10,000 × g for 20 min. The supernatant obtained (termed low-speed supernatant) was passed through a thin pad of glass wool to trap lipid particles.

Step II. Heat treatment: The low-speed supernatant was heated to 60° in a water bath maintained at 90–95°. After 3 min at 60° the enzyme solution was rapidly chilled and centrifuged in a 50 Ti rotor at 40,000 rpm for 60 min in an L265B ultracentrifuge.

Step III. Acid fractionation: The supernatant was adjusted to pH 4.5 by dropwise addition of 5 M acetic acid and centrifuged at 30,000 rpm for 30 min as in step II. The supernatant obtained was adjusted to pH 7.0 and dialyzed overnight against 6 liters of distilled water. Acid fractionation should be performed as rapidly as possible; prolonged treatment results in considerable loss of enzyme activity.

Step IV. Phosphocellulose column chromatography: The dialyzed fraction was diluted with an equal volume of cold distilled water and adjusted to pH 5.7 by addition of 5 M acetic

Table 1. Purification of Fru-P_2ase from brain

Fraction	Total units*	Specific activity†	Total protein, mg	Recovery, %
Low-speed supernatant	11,700	0.026	450	100
Heated fraction	7,684	0.068	113	65
Acid fraction	7,680	0.160	48	65
Phosphocellulose eluate	5,600	8.0	0.7	48

* 1 unit = amount of enzyme causing hydrolysis of 1 nmol of Fru-P_2 per hr.

† μmol of P_i released/mg of protein per hr.

acid. The material was then loaded on a phosphocellulose column (1.1 × 16 cm) previously equilibrated with 10 mM acetate buffer, pH 5.7, and washed with 500 ml of 0.2 M acetate buffer, pH 5.8, containing 0.1 mM EDTA. The enzyme was then eluted with 2 mM Fru-P_2 in 0.2 M acetate buffer, pH 6.4, containing 0.1 mM EDTA. Fractions of 5 ml were collected at 12-min intervals. Aliquots from different fractions were assayed for Fru-P_2ase activity. Fractions containing highest activity were pooled, adjusted to pH 7.6 by Tris, and incubated at 37° for 40 min for hydrolysis of the bound Fru-P_2, a procedure similar to that described by Taketa et al. (11). The solution was then placed in a dialysis sac and covered with dry Sephadex G-200 to reduce volume. The concentrated enzyme (about 2 ml) was dialyzed against 1 liter of 10 mM Tris·HCl buffer, pH 7.0, and stored frozen at −20°.

Standard disc gel electrophoresis was carried out in 7% acrylamide gels (12); and disc gel electrophoresis was in 0.1% sodium dodecyl sulfate (NaDodSO$_4$), according to Weber and Osborn (13), in 5% acrylamide gels. For NaDodSO$_4$ gel electrophoresis, protein samples were heated in a solution of 10 mM phosphate buffer, pH 7.0, containing 1% NaDodSO$_4$/0.5 M urea/1% mercaptoethanol in a boiling-water bath for 30 min before they were placed on the gel. Gels were stained overnight in 0.05% Coomassie brilliant blue/45% methanol/9% acetic acid (vol/vol) and destained by soaking in repeated changes of 7.5% acetic acid/5% methanol (vol/vol). Gels were stored in 7% formalin.

For preparation of antiserum against brain Fru-P_2ase, 3 mg of phosphocellulose-purified enzyme, emulsified with an equal volume of Freund's complete adjuvant (Difco) was injected into sheep, followed 2 weeks later by a similar injection. The animal was bled 5 weeks after the first injection and the serum was separated from the clot by centrifugation at 10,000 × g for 30 min. The undiluted serum was adjusted to 40% saturation with ammonium sulfate. The precipitate formed was dissolved in the minimum volume of 0.9% buffered saline, pH 7.6, and dialyzed exhaustively against the same solution. Protein was estimated by the procedure of Lowry et al. (14).

Crude extracts of liver and kidney containing 0.5 unit of Fru-P_2ase/ml were prepared according to Tejwani et al. (15). (One unit of enzyme is the amount causing hydrolysis of 1 nmol of Fru-P_2 per hr.) Purified rat liver Fru-P_2ase and its antiserum produced in rabbits were gifts from B. L. Horecker, Roche Institute of Molecular Biology.

RESULTS

Purification

Table 1 shows the results of a typical purification procedure for the brain Fru-P_2ase. From 12 g (wet weight) of brain tissue
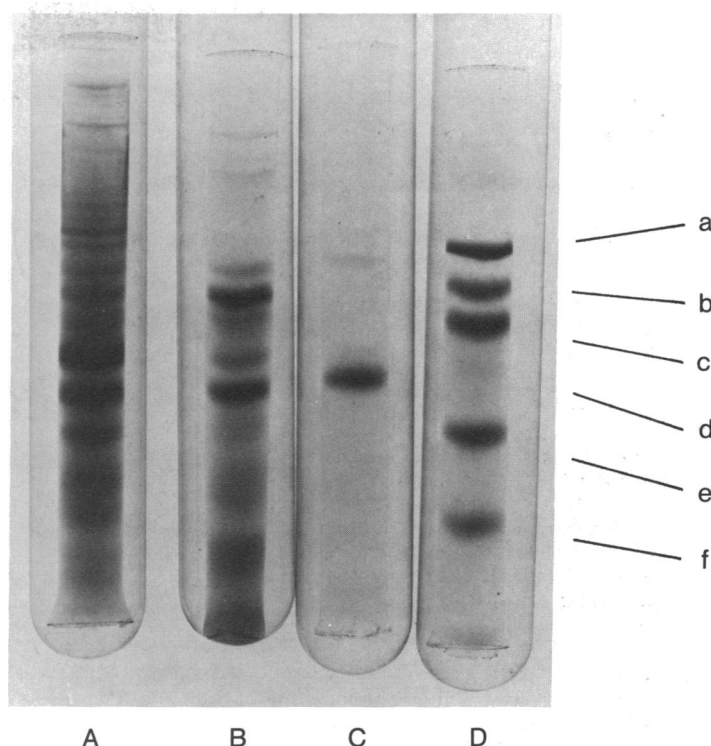

FIG. 1. NaDodSO$_4$/polyacrylamide gel electrophoresis of brain Fru-P_2ase at different steps of purification. (A) Protein (30 μg) from low-speed supernatant fraction; (B) 25 μg of protein, acid fraction; (C) 20 μg of protein, phosphocellulose fraction. (D) Marker proteins (molecular weight): (a) phosphorylase b (96,000); (b) transferrin (76,000); (c) glutamic dehydrogenase (55,000); (d) aldolase (40,000); (e) carbonic anhydrase (29,000); and (f) myoglobin (17,000).

about 0.7 mg of enzyme protein was recovered (48% of the original activity) purified 300-fold.

Purity

The phosphocellulose-purified brain Fru-P_2ase, on polyacrylamide gel electrophoresis in the presence of NaDodSO$_4$, showed one major protein band and two other protein bands of higher molecular weight (Fig. 1C). From the density of the individual protein bands, obtained by scanning the gel in a Gilford gel-scanner (kindly performed by Itsu Kano), the minor bands were calculated to constitute about 5% of the total peak area, indicating about 95% purity of the enzyme protein. By comparison with standard proteins of known molecular weight (Fig. 1D), the subunit molecular weight of the brain Fru-P_2ase was estimated at about 40,000. The enzyme activity was found to correspond to the major protein band when slices were assayed after electrophoresis in a standard 7% acrylamide gel without NaDodSO$_4$. Fig. 1 A and B shows the course of purification prior to phosphocellulose column chromatography.

Effect of pH, divalent cation, 5'-AMP, and phospholipid on catalytic activity

In the absence of added divalent cation, brain Fru-P_2ase has been found to be optimally active in a broad range between pH 7.6 and 9.5 (Fig. 2). Addition of EDTA or the acetates of Mg^{2+}, Ca^{2+}, Co^{2+}, and Mn^{2+} at 1.5 mM had no effect on the activity. Cu^{2+} and Zn^{2+} inhibited 48 and 28%, respectively. 5'-AMP, a potent inhibitor of liver, kidney, and mammalian muscle Fru-P_2ase (1), was without effect on the brain enzyme when tested between 0.05 and 0.5 mM. Phosphatidylcholine at a concentration of 1 mg/ml failed to stimulate the enzyme.
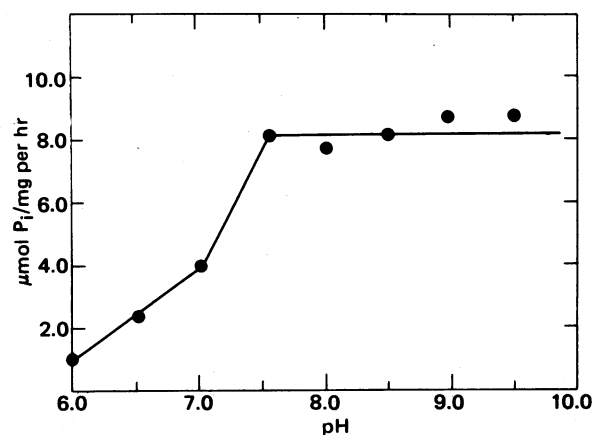
FIG. 2. Effect of pH on the catalytic activity of brain Fru-P_2ase. Phosphocellulose-purified enzyme (5 μg) was incubated as described in *Materials and Methods* in Tris-maleate (pH 6.0, 6.5) or Tris·HCl (pH 7.0–9.5) buffers. Fru-P_2ase activity was measured by determination of released P_i.

Substrate specificity and K_m

The purified Fru-P_2ase has been found to be highly specific for its substrate, Fru-P_2. Of the several other phosphate compounds tested as substrates at a concentration of 0.12 mM, i.e., β-glycerophosphate, glucose 6-phosphate, fructose 6-phosphate, glucose 1-phosphate, fructose 1-phosphate, glucose 1,6-bisphosphate, glycerol 1,3-bisphosphate, and sedoheptulose 1,7-bisphosphate, about 20% of the Fru-P_2ase activity was recorded with the last compound only. The enzyme failed to hydrolyze the other phosphate esters. K_m for the substrate, determined from a Lineweaver–Burk plot, was estimated to be approximately 1.32×10^{-4} M.

Immunologic properties of brain Fru-P_2ase

An interesting property of the brain Fru-P_2ase is its failure to react with antibody prepared against liver Fru-P_2ase, although muscle and kidney Fru-P_2ase react with such antibody preparations (15). Fig. 3 shows the results of studies made by the Ouchterlony double-diffusion technique. When crude Fru-P_2ase preparations from liver and kidney and purified Fru-P_2ase from brain were challenged by rabbit antiserum to liver Fru-P_2ase, precipitin bands were observed with liver and
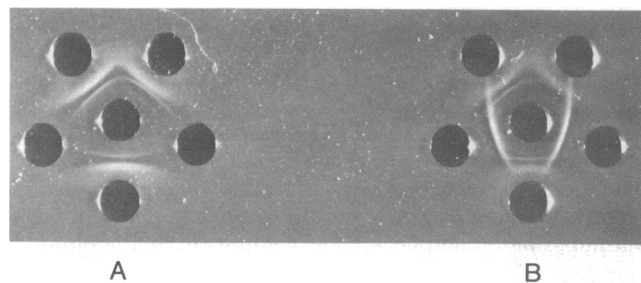

FIG. 3. Ouchterlony double-diffusion patterns obtained with (A) rabbit antiserum against liver Fru-P_2ase and (B) sheep antiserum against brain Fru-P_2ase. (A) The center well contained 5 μl of antiserum obtained from rabbits immunized with purified rat liver Fru-P_2ase. The outer wells (from bottom, clockwise) contained: first and third, 5 μl of crude liver Fru-P_2ase; fourth, 5 μl of crude kidney Fru-P_2ase; second and fifth, 10 μl of phosphocellulose fraction of brain Fru-P_2ase (1.4 mg/ml). (B) Center well contained 5 μl of 40% saturated ammonium sulfate fraction of antiserum obtained from sheep immunized with phosphocellulose-purified brain Fru-P_2ase. Outer wells contained different enzyme fractions as in A. Incubated at room temperature for 36 hr.
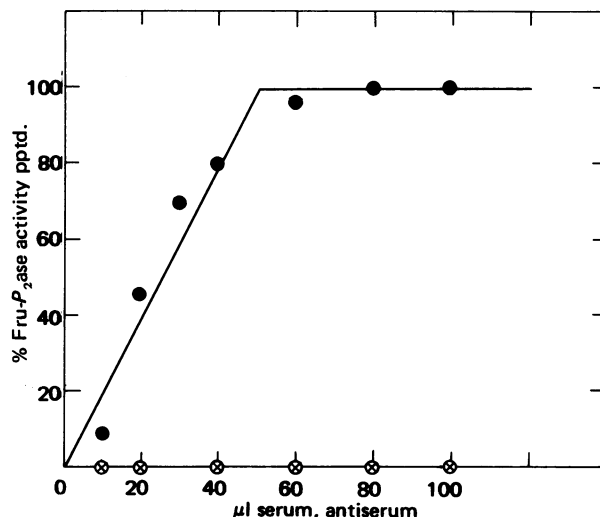

FIG. 4. Precipitation of brain Fru-P_2ase activity by sheep antiserum against brain Fru-P_2ase. Aliquots of brain Fru-P_2ase (each containing 32 units) were mixed with increasing amounts of sheep antiserum against brain Fru-P_2ase (●), sheep preimmunization control serum (O), or rabbit antiserum against liver Fru-P_2ase serum (×). To provide a precipitate for the latter two sets, an unrelated immuneprecipitate consisting of 25 μl of rabbit gamma globulin (5 mg/ml) and 50 μl of sheep antibody against rabbit gamma globulin was added to each of the tubes. After incubation for 1 hr at 37°, followed by incubation overnight at 4°, samples were centrifuged at 2000 rpm for 20 min and the precipitate was washed twice with 2 ml of 0.05 M Tris·HCl buffer, pH 7.6. The precipitate was assayed for Fru-P_2ase activity by determination of released P_i as described in *Materials and Methods*.

kidney fractions only but not with the purified brain enzyme (Fig. 3A). The precipitin bands consisted of a major outer band and a minor inner band, although only the major band was observed in separate experiments using purified liver enzyme (not shown).

Fig. 3B shows the results of similar experiments using antiserum produced in sheep against the purified brain enzyme. Only the brain Fru-P_2ase crossreacts with the antiserum. The fainter precipitin lines with the liver and kidney enzymes suggest the presence of other antibodies in the antiserum against brain Fru-P_2ase.

Preliminary experiments suggested that the brain Fru-P_2ase forms a precipitate when incubated with its antiserum and, moreover, that the enzyme–antibody complex is catalytically active. The results of the Ouchterlony double-diffusion experiments described above were thus confirmed by enzyme precipitation experiments, as illustrated in Fig. 4. Brain Fru-P_2ase was found to be quantitatively precipitated by its antiserum, but not by the preimmunization serum or by the rabbit antiserum against liver Fru-P_2ase. Thirty-two units of brain Fru-P_2ase were completely precipitated by 50 μl of the antiserum against brain Fru-P_2ase. Although an equivalent amount of liver Fru-P_2ase activity can be precipitated by 1 μl of the antiserum against liver Fru-P_2ase (15), 100 times as much antiserum against liver Fru-P_2ase was ineffective in precipitating or neutralizing the brain enzyme.

DISCUSSION

The present report is an unequivocal demonstration of a specific Fru-P_2ase in mammalian brain, a subject of long controversy (3, 16). To this end, Fru-P_2ase has been isolated from brain to 95% homogeneity, with a purification of 300-fold. Assuming no activation or inactivation of the enzyme during isolation,

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calculations from the data in Table 1 reveal that Fru-P_2ase comprises about 0.32% of the total protein in the brain low-speed supernatant (about 1.4 mg in 450 mg of low-speed supernatant protein). This value is similar to the 0.36% reported for the chicken liver enzyme (17). The purified brain enzyme catalyzes the hydrolysis of 0.13 μmol of Fru-P_2/min per mg of protein at pH 7.6 compared with a value of 29 μmol reported for the liver enzyme (15). The low gluconeogenic activity of brain compared with liver (16) may in part be a reflection of this lower specific activity of Fru-P_2ase rather than any dearth of enzyme.

Compared to the liver enzyme, the brain enzyme has similar subunit molecular weight, higher K_m for Fru-P_2, and identical substrate specificity; bovine liver Fru-P_2ase differs in exhibiting activity towards β-glycerophosphate (18). With respect to the lack of inhibition by 5'-AMP, the brain Fru-P_2ase is different from liver, kidney, and mammalian muscle enzymes but similar to the Fru-P_2ase of flight muscle of bumblebee (19). A further striking difference between Fru-P_2ase from brain and other tissues is the absence of a metal requirement for activity. No divalent cation is associated with the enzyme, as indicated by the failure of EDTA to inhibit, nor did the addition of Mg^{2+}, Ca^{2+}, Mn^{2+}, or Co^{2+} stimulate.

An important property of the brain enzyme is its immunological difference from the liver enzyme, because the former fails to crossreact with the antibody to purified rat liver enzyme (Fig. 3A). To what extent structure contributes to the immunological differences between the two enzymes must await further investigation. No immunological comparison of brain and muscle enzymes has been made.

The search for Fru-P_2ase in brain was prompted by an earlier study which showed that the addition of phospholipid to brain supernatant caused the accumulation of glucose 6-phosphate (5). The effect was localized at the level of the fructose phosphates of the glycolytic cycle. Since stimulation of Fru-P_2ase could account for the accumulation of glucose 6-phosphate and since, moreover, we had observed in the same study the conversion of [^{14}C]Fru-P_2 into [^{14}C]fructose 6-phosphate, we undertook the search for this enzyme. Although we have demonstrated unequivocally that brain contains Fru-P_2ase, further study has shown that it is not the enzyme to which the inhibitory effect of phospholipid on glycolysis can be attributed. This was shown both by the inertness of the purified enzyme toward phosphatidylcholine and the lack of any increase in labeled inorganic phosphate from [1-^{32}P]Fru-P_2 under conditions in which phosphatidylcholine causes glucose 6-phosphate to accumulate. The responsible enzyme then must be phosphofructokinase, which in other tissues is in fact inhibited by phospholipid (20). Whether the brain phosphofructokinase (21) is also inhibited by phospholipid awaits further study.

Our unequivocal demonstration of Fru-P_2ase in brain provides a direct pathway from Fru-P_2 to fructose 6-phosphate and, together with phosphofructokinase, the potential for a hitherto unknown fructose phosphate "futile cycle" in brain, a possible control point in the distribution of glucose 6-phosphate between glycolysis and other pathways of utilization.

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