Role of Inosine 5′-Phosphate in Activating Glucose-bisphosphatase†

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ABSTRACT: Glucose-bisphosphate (Glc-1,6-P_2) phosphatase has been purified >200-fold from the cytosol of mouse brain. As reported earlier, the enzyme requires inosine monophosphate (IMP) and Mg^{2+} for activity [Guha, S. K., & Rose, Z. B. (1982) *J. Biol. Chem. 257*, 6634-6637]. Kinetic parameters and the role of IMP have been further investigated. When Glc-1,6-P_2 and IMP are both varied, double-reciprocal plots of the data form a parallel line pattern. With 2 mM Mg^{2+}, the K_m obtained for Glc-1,6-P_2 is 20 μM and the K_a for IMP is 9 μM. Co^{2+}, Mn^{2+}, and Ni^{2+} activate less effectively than Mg^{2+}. The apparent K_a for Mg^{2+} decreases with increasing Glc-1,6-P_2, and the observed K_m of Glc-1,6-P_2 decreases with increasing Mg^{2+}. The extrapolated value of the K_a of Mg^{2+} at infinite substrate is 86 μM. Mg^{2+} does not affect the K_a of IMP. The phosphatase activity is optimal at pH 7. The phosphatase is not completely specific since mannose 1,6-bisphosphate is hydrolyzed and guanosine monophosphate activates. However, fructose 1,6-bisphosphate is no more than a poor inhibitor, and adenine nucleotides are neither activators nor inhibitors. The products of the reaction are glucose-1-P and glucose-6-P, in a ratio of 2:3, and P_i. Both glucose-P's are competitive inhibitors with respect to IMP [K_i(glucose-1-P) = 5 μM; K_i(glucose-6-P) = 18 μM]. Neither glucose-P competes with Glc-1,6-P_2. The demonstration of an exchange reaction between Glc-1,6-P_2 and glucose-6-P is evidence for the phosphorylation of the enzyme by the substrate. The exchange reaction requires Mg^{2+} and is inhibited by IMP. The observation of the exchange reaction and its elimination by IMP indicates that the low level of phosphoglucomutase activity that remains with the phosphatase throughout purification is an inherent property of the phosphatase. The requirement of glucose-bisphosphatase for the nucleotide IMP is consistent with possible roles for both Glc-1,6-P_2 and IMP in the control of the ATP level in the brain.

Glucose bisphosphate (Glc-1,6-P_2)[1] is well-known as the activator of phosphoglucomutase (Leloir et al., 1948). However, the level of Glc-1,6-P_2 varies over a 10-fold range in different mouse tissues and does not correlate with the phosphoglucomutase activity of a given tissue (Passonneau et al., 1969). Brain and red blood cells have the highest concentrations of Glc-1,6-P_2, close to 100 μM. The bisphosphate can modify the activities of several enzymes that are important regulators of carbohydrate metabolism, including hexokinase (Crane & Sols, 1954; Gerber et al., 1974; Rose et al., 1974; Beitner et al., 1975), phosphofructokinase (Hofer & Pette, 1968; Rose & Warms, 1974), pyruvate kinase (Koster et al., 1972), fructose-bisphosphatase (Marcus, 1976), and 6-P-gluconate dehydrogenase (Beitner & Nordenberg, 1979). In brain, Glc-1,6-P_2 was observed to break down rapidly under ischemic conditions, indicating that it is metabolically active (Passonneau et al., 1969). An enzyme specific for the synthesis of Glc-1,6-P_2 has been purified and found to be sensitive to inhibition by physiologically significant levels of fructose-1,6-P_2, 3-phosphoglycerate, and citrate (Rose et al., 1975, 1977; Wong & Rose, 1976). These observations lend interest to additional studies of metabolic interactions of Glc-1,6-P_2.

Recently we reported the occurrence in brain of a specific phosphatase for Glc-1,6-P_2 (Guha & Rose, 1982). This cytosolic enzyme requires a soluble, heat-stable factor which was identified as inosinic acid (IMP). AMP neither activates nor inhibits. The glucose-bisphosphatase activity was separated from phosphoglucomutase and partially separated from glucose-bisphosphate synthase by ion-exchange chromatography. The present paper reports on the properties of the phosphatase and considers the role of IMP in the reaction.

Materials and Methods

Materials. DEAE-cellulose, fibrous, was from Whatman. Blue Sepharose CL-6B, Sephacryl S-200, and DEAE-Sephacel were from Pharmacia. Carbowax [poly(ethylene glycol), M_r 20000] was from Fisher Scientific Co. IMP, GMP, ATP, AMP, glucose bisphosphate, bovine serum albumin (fraction V), cytochrome *c*, lactate dehydrogenase, and phosphoglucomutase were from Sigma. IDP (Calbiochem) was purified by ion-exchange chromatography to remove contaminating IMP. Enolase and glucose-6-P dehydrogenase were from Boehringer. Carbonic anhydrase was from Worthington Biochemical Corp. [^{32}P]Phosphoric acid, carrier free, was from New England Nuclear. Mannose bisphosphate was synthesized according to Pasternak (1953) by Drs. Robert K. Preston and Richard M. Peck of this institute.

A Packard TriCarb scintillation counter was used for Cerenkov counting when possible or with Liquifluor (New England Nuclear) in toluene.

[U-^{32}P]Glc-1,6-P_2 was synthesized enzymatically as described earlier (Guha & Rose, 1982). Glc-6-[^{32}P]P was prepared by hydrolysis of [U-^{32}P]Glc-1,6-P_2 in 0.5 N HCl at 100 °C for 20 min.

Distribution of Enzyme in Brain. An extract was prepared in a Dounce homogenizer from the brains of two 2-month-old mice, 1 g total weight, by using 5 mL of buffer containing 10 mM TEA-Cl^--1 mM EDTA-2 mM mercaptoethanol-0.25 M sucrose, pH 7.1. After centrifugation at 180*g* for 5 min to remove unbroken cells, the extract was fractionated according to the procedure of Fleischer & Kervina (1974). Before assay each fraction was passed through a column of Sephadex G-25 equilibrated with buffer lacking sucrose.

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[1] Abbreviations: TEA, triethanolamine; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; Glc-1-P, glucose 1-phosphate; Glc-6-P, glucose 6-phosphate; Glc-1,6-P_2, glucose 1,6-bisphosphate; *V*, maximal velocity; IMP, inosine 5′-phosphate.

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Protein was determined according to Lowry et al. (1951).

Enzyme Assays. Phosphate release from [U-^{32}P]Glc-1,6-P_2 was measured after incubation at 37 °C in a 0.2-mL volume in 1-mL conical plastic tubes containing 50 mM Tes–Na^+ buffer, pH 7.3, 2 mM mercaptoethanol, 0.11 mg of bovine serum albumin, 2 mM $MgCl_2$, 100 μM IMP, 50 μM [U-^{32}P]Glc-1,6-P_2 containing (5–6) × 10^4 cpm, and enzyme sufficient to convert not more than 15% of the substrate to products. The reaction was stopped by the addition of 0.05 mL of 50% trichloroacetic acid. After brief centrifugation, a 0.2-mL sample was removed to which was added 0.05 mL of 4 N H_2SO_4 containing 3 mM K_2HPO_4, 0.05 mL of 5% ammonium molybdate, and 0.6 mL of isobutyl alcohol (water saturated) (Berenblum & Chain, 1938). After 25 s on a junior Vortex mixer, the tube was centrifuged briefly and 0.3 mL of the isobutyl alcohol phase was counted in a scintillation counter. One unit of enzyme catalyzes the hydrolysis of 1 μmol of substrate/min under the conditions of the standard assay.

Color Test for P_i Release. P_i released during hydrolysis could be measured by using malachite green in acid–molybdate (Itaya & Ui, 1966; Toshima & Yoshimura, 1975). The incubations were for 5 min and contained 0.015 mg of albumin. Equal volumes of 3% $(NH_4)_6MoO_{24}{\cdot}4H_2O$ in water and 0.06% malachite green in 6 N HCl were mixed daily. The reactions were stopped by addition of 0.1 mL of reagent to a 0.2-mL incubation. Standards were run in the same medium as the unknown since interference by the albumin in the assays and also by trichloroacetic acid was observed.

Products of the Hydrolysis of Glc-1,6-P_2. The production of Glc-6-P and/or Glc-1-P was observed spectrophotometrically. Glc-6-P was detected with Glc-6-P dehydrogenase by observing the change in A_{340nm} from the reduction of NADP. Both Glc-6-P and Glc-1-P were detected in a second cuvette that was supplemented with phosphoglucomutase. The Glc-1,6-P_2 in the incubations was labeled with [^{32}P]P_i; samples were withdrawn periodically from each cuvette and extracted for [^{32}P]P_i or P_i was assayed directly by using the molybdate–malachite green reagent. Each cuvette contained in 0.45 mL 60 mM Tes–Na^+ buffer, pH 7.3, 2 mM mercaptoethanol, 1 mM EDTA, 0.3 mg of bovine serum albumin, 4 mM $MgCl_2$, 0.1 mM IMP, 0.24 mM NADP, 0.079 mM [U-^{32}P]Glc-1,6-P_2 (1.16 × 10^5 cpm/10^{-6} atom ^{32}P), and 1.68 units of Glc-6-P dehydrogenase. The second cuvette also contained 5.4 units of phosphoglucomutase. The reactions were started with glucose-bisphosphate phosphatase.

Dissociation Constants and pK Values. For Glc-1,6-P_2, Ray & Roscelli (1966) determined the pK_a values for the third and fourth ionization constants as 6.0 and 6.8 as compared to 6.1 for the second ionization in a mixture of Glc-1-P and Glc-6-P. They concluded that there are no significant bidentate binding effects involving the two phosphate groups of Glc-1,6-P_2. Dissociation constants for Mg^{2+} complexes of Glc-1,6-P_2 were reported as a function of ionic strength. From their data, the value for the dissociation constant of the Mg^{2+} complex of Glc-1,6-P_2 under the conditions of the present study would be 1.55 × 10^{-3} M. Since the two phosphate groups bind Mg^{2+} independently, this is a combined dissociation constant and the true K_d for each site should be twice that value or about 3 mM. This agrees with data from this laboratory (unpublished) in which, in collaboration with Dr. G.D. Markham, it was found by using electron paramagnetic resonance (EPR) that Glc-1,6-P_2 has two independent binding sites for Mn^{2+}. The binding of Mg^{2+} to Glc-1,6-P_2, determined by competition with Mn^{2+}, is consistent with K_d = 3 mM. The binding of Mg^{2+} to glycerate-2,3-P_2 was measured in this way by Gupta & Benovic (1978), who found K_d = 3 mM for each of two independent sites on that compound. The binding of Mg^{2+} to IMP is very weak, K_d = 10 mM (Sillén et al., 1971).

Table I: Distribution of Phosphatase Activity in the Brain[a]

fraction	phosphatase activity: nmol min^{-1} (mg of protein)$^{-1}$ (–)IMP	(+)IMP	nmol min^{-1} (g of brain)$^{-1}$ (–)IMP	(+)IMP
homogenate	0.20	0.45	18	40
nuclei, plasma membranes	0.20	0.34	12	19
mitochondria, lysosomes, peroxisomes	0.21	0.20	2.8	2.6
golgi, lysosomes	0.07	0.05	0.2	0.1
microsomes	0.03	0.03	0.1	0.1
cytosol	0.07	0.64	1.3	12

[a] See Materials and Methods for conditions.

Molecular Weight Determination. The molecular weight of the enzyme was determined by gel filtration (Andrews, 1964) on a column of Sephacryl S-200 (0.69 × 86.5 cm; volume 130 mL) equilibrated with 10 mM TEA–Cl^-–1 mM EDTA–2 mM mercaptoethanol, pH 7.3. The column was calibrated with the following standards (M_r): muscle lactate dehydrogenase (135 000); muscle enolase (82 000); bovine erythrocyte carbonic anhydrase (30 000); human hemoglobin (64 500); horse heart cytochrome *c* (12 500). Lactate dehydrogenase and enolase were located by their activity; carbonic anhydrase was located by its absorbance at 280 nm; hemoglobin and cytochrome *c* were monitored at 540 nm. The void volume was determined with ferritin which was monitored at 280 nm.

Results and Discussion

Distribution of Enzyme in Brain. A homogenate of mouse brain was fractionated to separate the various organelles. Glucose-bisphosphatase activity was assayed in each fraction with and without IMP (Table I). A large part of the phosphatase activity was in the particulate fraction containing plasma membranes and nuclei. This activity was enhanced 1.5-fold by the addition of IMP. Most of the remaining activity was in the cytosol, in which case the stimulation by IMP was 9-fold. The IMP-activated phosphatase in the cytosol is the subject of this study.

Enzyme Purification. When a DEAE-cellulose column eluted at pH 7.3 with a gradient of KCl was used, phosphoglucomutase emerged very early followed by glucose-bisphosphate synthase (peak at 25 mM Cl^-) and glucose-bisphosphate phosphatase (peak at 175 mM). However, the separation between the synthase and phosphatase was not complete. In the following preparation an attempt was made to optimize the conditions for the separation of these activities from the phosphatase. All steps were carried out at 0–4 °C.

(*1*) *Tissue Extraction.* Fifty male mice (ICR strain) 4–5 weeks old were killed by decapitation. The brains were removed, washed in 10 mM TEA–Cl^- buffer, pH 7.2, containing 0.25 M sucrose, 1 mM EDTA, and 2 mM mercaptoethanol (buffer A), and homogenized in 4 volumes of the same buffer. The extract was obtained after centrifugation at 37000*g* for 1 h.

(*2*) *DEAE-cellulose Chromatography.* The cytosol was applied to a column (3.5 × 9 cm) of Eastman fibrous DEAE-cellulose equilibrated with 10 mM TEA–Cl^- buffer, pH 7.3, containing 1 mM EDTA and 2 mM mercaptoethanol (buffer B). The column was washed with 500 mL of the same

Table II: Purification of Glucose-bisphosphate Phosphatase

fraction	vol (mL)	act. (milliunits)	protein (mg)[a]	sp act. (milliunits/mg)	+IMP/–IMP	phosphatase/ PGM[b]
extract	80	254	1208	0.21	2	
DEAE-cellulose	32	337	85	3.96		812
DEAE-Sephacel	11	298	14	21.3		1777
blue dextran	5	161	3.4	47.3	37	1811

[a] From A_{280nm} when an extinction coefficient of 10 is assumed for a 1% solution. [b] Phosphoglucomutase.

buffer and then with 500 mL of buffer containing 0.08 M KCl. When a linear gradient formed from 300 mL each of buffer containing 0.08 M KCl or 0.4 M KCl was used, the enzyme eluted with 140–230 mM Cl^-. The active fractions were combined in a dialysis bag and concentrated to 80 mL overnight in Carbowax.

(*3*) *Chromatography on DEAE-Sephacel.* The enzyme fraction was diluted to 200 mL with buffer B and applied to a column of DEAE-Sephacel (2.5 × 4 cm) equilibrated with buffer B. The column was washed with 100 mL of equilibration buffer containing 0.1 M KCl and eluted with a linear gradient formed from 75 mL each of the same buffer or buffer containing 0.2 M KCl, followed by 50 mL of the latter solution. The enzyme started to elute with 0.13 M KCl. The active fractions were combined and concentrated by ultrafiltration in an Amicon chamber with a YM-10 membrane.

(*4*) *Fractionation on Blue Dextran Sepharose.* A column (2.5 × 5 cm) of blue Sepharose CL-6B was equilibrated with 10 mM TEA–Cl^-, pH 7.3, containing 0.5 mM EDTA, 2 mM $MgCl_2$, and 2 mM mercaptoethanol. The fraction from the previous step was diluted to decrease the chloride concentration to 10–15 mM and applied to the column. The column was washed with 100 mL of equilibration buffer and 50 mL of buffer containing 0.1 M KCl. The enzyme was eluted with a gradient derived from 100 mL each of the latter buffer or buffer with 0.2 M KCl. The enzyme eluted with 100 mM Cl^- and higher. The active fractions were combined and concentrated to 10.5 mL (0.7 mg of protein/mL) by ultrafiltration in an Amicon chamber.

The procedure achieved a purification of >200-fold (Table II). The phosphoglucomutase activity of the preparation was a particular concern. As mentioned, phosphoglucomutase itself separated readily from the phosphatase. In this preparation conditions were chosen to optimize the separation from glucose-bisphosphate synthase, which also has phosphoglucomutase activity. The final ratio of phosphatase to phosphoglucomutase activity achieved was 1800. Evidence to be presented indicates that this activity is an inherent property of the phosphatase.

Stability and Molecular Properties. The enzyme fractions could be stored with little loss of activity for 1–2 months at 4 °C or frozen at −80 °C. The enzyme lost activity during incubations unless supplemented with serum albumin at 0.55 mg/mL which allowed linear rates for at least 30 min.

The molecular weight of the phosphatase is ~87 000 as determined by gel filtration on Sephacryl S-200 using the standards and conditions described under Materials and Methods.

Kinetic Pattern. Glucose-bisphosphatase requires both IMP and Mg^{2+} for optimal activity (Guha & Rose, 1982). In the crude extract IMP stimulates glucose-bisphosphatase activity 2-fold under the standard assay conditions. The activity of the purified enzyme, assayed under the same conditions, decreased to 2% of the activated rate. Without IMP, the K_m determined for Glc-1,6-P_2 was 150 μM and the maximal velocity was about 8% of that obtained in the presence of IMP.

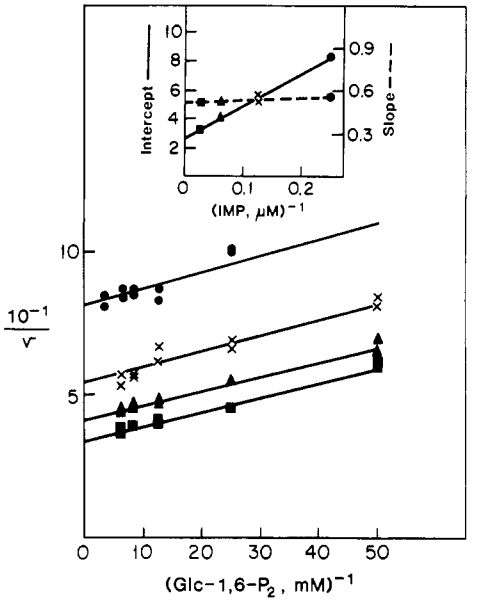

FIGURE 1: Activation by IMP. The incubations were for 25 min under the conditions given under Materials and Methods for the enzyme assay. $MgCl_2$ was 2 mM. Glc-1,6-P_2 was varied by adding different amounts of carrier to a tracer amount of [U-^{32}P]Glc-1,6-P_2 containing 6.50 × 10^4 cpm/μatom ^{32}P. IMP was also varied (μM): 4 (●); 8 (×); 16 (▲); 32 (■). v is expressed as μmol min^{-1} (mL of enzyme)$^{-1}$. V was obtained from the replot of the intercepts.

It is not clear whether the IMP-dependent and -independent activities are due to the same enzyme, but the contribution of the IMP-independent reaction would be small in these studies.

IDP, purified by ion exchange to remove contaminating IMP, was neither an activator nor an inhibitor of the phosphatase reaction.

The kinetic pattern obtained by varying glucose bisphosphate and IMP is shown in Figure 1. The reaction follows Michaelis–Menten kinetics and gives a parallel line pattern. The K_a for IMP obtained from the replot of the intercepts is 9 μM. The K_m is 20 μM for Glc-1,6-P_2 as calculated from the slope of the lines and the expression slope = K_m/V.

Effects of Divalent Metal Ions. The phosphatase requires an added divalent cation for activity. Mg^{2+} activates more effectively than other cations that were tested. At 2 mM, which appeared to be optimal in all cases, the relative activities were (%) 100 for Mg^{2+}, 35 for Co^{2+}, 12 for Mn^{2+}, and 4 for Ni^{2+}. Cd^{2+}, Ca^{2+}, and Zn^{2+} (0.1–2 mM) do not activate. In the presence of 1 mM Mg^{2+}, 15 μM EDTA, and 50 μM metal ion, all of the divalent cations tested inhibit appreciably (% inhibition): Cd^{2+} (92), Cu^{2+} (92), Zn^{2+} (85), Ca^{2+} (59); Co^{2+} (44), Ni^{2+} (40), Mn^{2+} (38), and Be^{2+} (11). When Mg^{2+} was limiting, EDTA was inhibitory, whereas with higher Mg^{2+}, EDTA increased the phosphatase rate. Therefore, the phosphatase may bind some inhibitory metal ions tightly. There was no effect of 20 mM LiCl, which is inhibitory in the micromolar range for both phosphoglucomutase (Ray et al.,

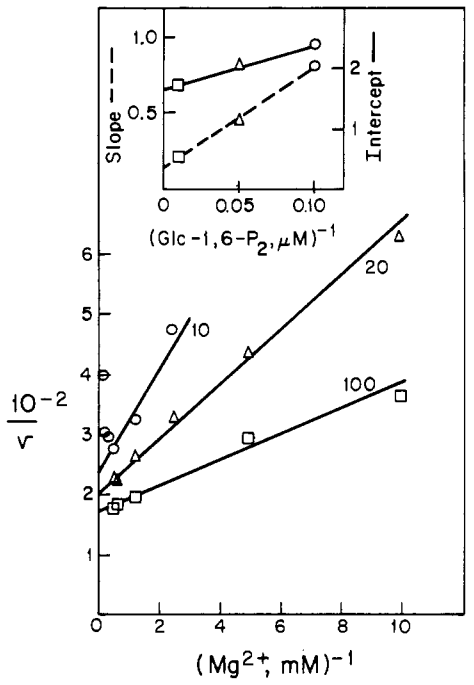

FIGURE 2: Effects of varying Mg^{2+} and glucose bisphosphate. The incubations contained 100 μM IMP and the indicated concentrations of Mg^{2+} at three levels of [U-^{32}P]Glc-1,6-P_2 (μM): 10 (○); 20 (△); 100 (□). Other conditions were as in Figure 1. The amount of enzyme was constant, but the incubation time was varied from 10 to 30 min in order to keep the extent of reaction within the desired limits.

1978) and glucose-bisphosphate synthase (Rose et al., 1975). Be^{2+} is a potent inhibitor and inactivator of phosphoglucomutase (Aldridge & Thomas, 1966; Hashimoto et al., 1967). The phosphatase activity and the phosphoglucomutase activity associated with it were not affected by preincubation with 25 μM Be^{2+} under conditions that gave 86% inhibition of muscle phosphoglucomutase in a parallel experiment. Thus, the phosphoglucomutase activity of the phosphatase preparation is not due to phosphoglucomutase itself.

The effect of varying Mg^{2+} and Glc-1,6-P_2 is shown in Figure 2. The apparent K_a for Mg^{2+} decreases with increasing Glc-1,6-P_2 and the observed K_m of Glc-1,6-P_2 decreases with increasing Mg^{2+}. From the data in Figure 2, the extrapolated value of the K_a of Mg^{2+} at infinite substrate is 86 μM. Mg^{2+} does not affect the K_a of IMP (data not shown). This behavior is compatible with Glc-1,6-P_2 acting as its Mg complex. However, it has been pointed out that if the ternary complex of enzyme, substrate, and Mg^{2+} forms rapidly relative to a subsequent step, it is not possible to learn anything about the steps involved in the formation of the complex by steady-state kinetic studies (Wimmer & Rose, 1978).

Very high levels of Mg^{2+} are inhibitory. With 10 μM Glc-1,6-P_2, Mg^{2+} above 2 mM is inhibitory (Figure 2). At higher Glc-1,6-P_2 levels, more Mg^{2+} is needed to observe inhibition.

Products of the Reaction. Previously we found that half of the counts in [U-^{32}P]Glc-1,6-P_2 could be released as P_i during the enzymatic reaction and that neither Glc-1-P nor Glc-6-P was hydrolyzed under the same conditions (Guha & Rose, 1982). The reaction appears to be a simple hydrolytic one with no evidence for the transfer of a phosphoryl group to IMP as shown by the failure to accumulate any ^{32}P-containing charcoal bound product during the course of the reaction. The products of the reaction were now identified more definitively. Glc-6-P formation was detected by enzymatic assays coupled to NADP reduction catalyzed by glucose-6-P dehydrogenase. The sum of Glc-1-P and Glc-6-P produced was obtained when phosphoglucomutase was present in the assay system. Inorganic phosphate produced in the same incubations was also monitored. Details of the experiment are under Materials and Methods and the results are summarized in Table III. The data indicated that the reaction produces Glc-1-P and Glc-6-P in a ratio of about 2:3. When the reaction had gone to completion, phosphoglucomutase was added to the cuvette previously lacking it. An immediate rapid increase in the optical density indicated that Glc-1-P had accumulated in that cuvette. It will be shown that the endogenous phosphoglucomutase activity is not functional in the presence of IMP. The same amount of P_i was produced in both cuvettes, and it was equal to the sum of the Glc-P's produced and the total Glc-1,6-P_2 consumed in the reaction.

Table III: Products of the Hydrolysis of Glucose Bisphosphate[a]

time (min)	Glc-6-P (nmol)	Glc-1-P + Glc-6-P (nmol)	P_i (nmol)	% of products as Glc-6-P
10	5	7.5	6.7	67
20	8.6	13.4	13.8	64
30	11.7	18.9	18.5	62
50	16.5	26.9	27.9	61
112	19.4	31.2	36.1	62
140	19.4	31.2	35.2	62

[a] The conditions of the experiment are given under Materials and Methods. The incubation contained 0.0357 μmol of Glc-1,6-P_2. The rate of P_i release was the same in the presence or absence of added phosphoglucomutase.

Effects of Glucose-1-P and Glucose-6-P. Glc-1-P and Glc-6-P have similar properties as inhibitors of the phosphatase reaction. When either is varied while also varying Glc-1,6-P_2, the double-reciprocal plots are a series of parallel lines, indicating that the products do not compete with Glc-1,6-P_2. Both Glc-1-P and Glc-6-P are competitive inhibitors with respect to IMP with K_i values of 5 μM and 18 μM, respectively.

Evidence for Phosphoenzyme Formation. Several pieces of data that have been presented suggest that there may be a phosphorylated enzyme intermediate in the phosphatase reaction. First, the parallel line kinetic pattern (Figure 1) is suggestive of the occurrence of a stable intermediate. Second, the observation that the inhibition by the products, Glc-1-P and Glc-6-P, is competitive with IMP and not with Glc-1,6-P_2 indicates that there are at least two forms of the enzyme with significantly different structures and the products do not interact well with the form of the enzyme that binds the substrate. Third, the finding that the phosphatase reaction produces both Glc-1-P and Glc-6-P and that the phosphatase appears to possess phosphoglucomutase activity could mean that the enzyme bears a mechanistic relationship to phosphoglucomutase, which is known to function with a phosphoenzyme intermediate (Anderson & Jolles, 1957; Ray & Peck, 1972). If the substrate does phosphorylate the enzyme, it might be possible to demonstrate an exchange reaction between the substrate and the first product:

$$E + \text{Glc-1,6-}P_2 \rightleftharpoons E\cdot\text{Glc-1,6-}P_2 \rightleftharpoons E\text{-}P + \text{Glc-6-P}$$

The reaction was carried out with unlabeled Glc-1,6-P_2 in the presence of Glc-6-[^{32}P]P. An exchange reaction was demonstrated (Table IV). The exchange required Mg^{2+} and was inhibited by IMP. Higher IMP completely eliminated exchange. IMP has no effect on phosphoglucomutase itself. Therefore, the phosphoglucomutase activity is a property of the phosphatase itself and not due to extraneous phospho-

Table IV: Demonstration of Exchange Reaction between Glc-6-[^{32}P]P and Glc-1,6-P_2[a]

IMP (μM)	counts in Glc-1,6-P_2 (cpm)	
	observed	per 60-min incubation
0	76 068	24 021
1	18 231	16 830
10	291	873

[a] Incubations at 37 °C in 0.2-mL volumes contained 50 mM Tes-Na^+ buffer, pH 7.3, 2 mM mercaptoethanol, 0.11 mg of serum albumin, 2 mM $MgCl_2$, 25 μM Glc-1,6-P_2, 50 μM Glc-6-[^{32}P]P (5.23×10^7 cpm/μmol), and enzyme. IMP was 0, 1, or 10 μM. In parallel incubations the phosphatase activity was assayed with the malachite green reagent and the remaining incubations were continued until about half of the substrate was hydrolyzed in each case [190 min (no IMP), 65 min (1 μM IMP), and 20 min (10 μM IMP)]. The reactions were stopped with 50 μL of 50% trichloroacetic acid. After centrifugation, 225 μL of supernatant was removed, neutralized with TEA base, diluted to 1 mL, and applied to a column of Dowex Cl^- (1 × 2.6 cm). Columns were washed with 10 mL of water and 50 mL of 20 mM HCl to remove P_i and monophosphates. The Glc-1,6-P_2 was eluted with 40 mM HCl. Fractions were collected and counted by the Cerenkov procedure. The total recovery of counts from each column was similar. The actual counts recovered in the Glc-1,6-P_2 region are given as well as values calculated for a 1-h incubation period. The counts from a control incubation without enzyme (2712 cpm) have been subtracted.

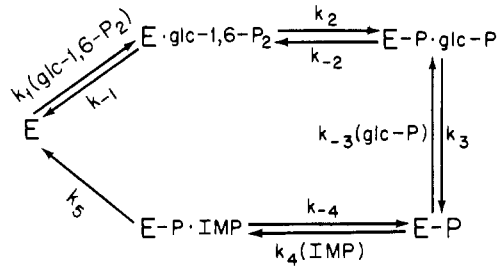

FIGURE 3: Proposed reaction scheme. The scheme has been simplified by the omission of Mg^{2+} as a reactant. E is enzyme; Glc-P is Glc-1-P or Glc-6-P.

glucomutase. The results are consistent with the scheme in Figure 3 in which the role of IMP is to assist in the release of the phosphoryl group from the enzyme.

As mentioned, Mg^{2+} is required for the exchange reaction. In order to learn whether Mg^{2+} also has a role in the phosphoryl transfer step, the relative rates of the overall reaction and the exchange reaction were measured either with limiting Mg^{2+} (0.03 mM) or saturating Mg^{2+} (0.9 mM) in the presence of low IMP (3 μM). At the higher level of Mg^{2+}, the P_i formation increased ~3-fold and the exchange rate increased ~2-fold. Since Mg^{2+} affects the exchange and overall rates to roughly the same extent, it is not possible to conclude whether the transfer step requires Mg^{2+}.

Effects of Ions and Sulfhydryl Reagents. Glucose-bisphosphatase is sensitive to inhibition by a variety of salts. Tes-Na^+ buffer shows optimal activity at 0.1 M, with activity decreasing by 30% in 0.4 M buffer. The effects of monovalent ions do not appear to be specific but rather a function of the ionic strength; KCl and potassium acetate both inhibit about 25% at 0.1 M and 50% at 0.2 M. The divalent anions P_i and SO_4^{2-} are significantly more inhibitory than monovalent anions at the same ionic strength: 10 mM potassium phosphate (pH 7.2) inhibits 50% and 5 mM ammonium sulfate inhibits 65%.

Inhibition was observed with a number of reagents that react with sulfhydryl groups: *p*-(hydroxymercuri)benzoate (5 μM) inhibited 87%, iodoacetate (5 mM) inhibited 25%, and iodoacetamide (5 mM) inhibited 40%.

Table V: Effects of Metabolites on Phosphatase Rate[a]

compound	concn	Mg^{2+} (mM)	act. (%)
AMP	0.1 mM	3	109
	0.2 mM	3	95
ADP	0.2 mM	3	99
	0.4 mM	3	91
ATP	2 mM	10[b]	122
	4 mM	10[b]	117
mannose-1,6-P_2	10 μM	2	58
	20 μM	2	37
fructose-1,6-P_2	100 μM	2	99
	200 μM	2	82
citrate	1 mM	5	90
	2 mM	5	95
glycerate-3-P	0.2 mM	5	89
	0.4 mM	5	87

[a] All incubations contained 10 μM Glc-1,6-P_2 and 3 μM IMP. Activity is compared to an incubation done under the same conditions without the test compound. [b] The control rate was 66% of the rate with 3 mM Mg^{2+}.

Effects of Metabolites on Phophatase Rate. A number of compounds were tested for possible effects on the phosphatase under conditions in which both Glc-1,6-P_2 and IMP were rate limiting. When present at much higher concentrations than IMP, adenine nucleotides were without effect on the phosphatase rate (Table V). The apparent activation by ATP is probably an artifact resulting from the inhibition of the enzyme in the control by excess Mg^{2+} which was countered by the complexation of Mg^{2+} by ATP.

Guanosine monophosphate (GMP) can activate in place of IMP. With GMP, the maximal velocity is about 60% of that with IMP. The K_a for GMP is 70 μM or 10 times higher than that of IMP under the same conditions. V/K for Glc-1,6-P_2 is 17 times higher for IMP than for GMP.

Citrate and glycerate-3-P and fructose-1,6-P_2 which are strong inhibitors of glucose-bisphosphate synthase (Rose et al., 1977) have little effect on the phosphatase. Mannose-1,6-P_2, which appears to be very active as an inhibitor, is actually an alternative substrate (see below).

Interactions of Mannose Bisphosphate. Mannose-1,6-P_2 is a good inhibitor of the reaction with Glc-1,6-P_2 (Table V). It is competitive with Glc-1,6-P_2 with an apparent K_i of 10 μM under conditions in which the apparent K_m of Glc-1,6-P_2 is 9 μM (low IMP).

Mannose-1,6-P_2 is also a substrate for the phosphatase. The hydrolysis rate was stimulated by 50 μM IMP. The K_i obtained as an inhibitor with respect to Glc-1,6-P_2 is really the K_m of mannose-1,6-P_2 under the conditions of the experiment. The K_m for mannose-1,6-P_2 determined directly in the presence of 50 μM IMP was 39 μM. The maximal velocities were compared with high levels of the two substrates (100 μM Glc-1,6-P_2 or 400 μM mannose-1,6-P_2) and IMP (75 μM) by using the malachite green color test to determine P_i release. The rate with mannose bisphosphate was about the same as that with glucose bisphosphate.

pH Dependence. The phosphatase activity is optimal at pH 7 under conditions that give maximal rates over the entire pH range. The rates at pH 6.0 and 8.4 were 70% and 50%, respectively, of the maximal rate. Above pH 7.5 the K_m of Glc-1,6-P_2 increases. The levels of IMP and Mg^{2+} required for maximal rates increase slightly between pH 6.6 and pH 8.0.

Summary

Kinetic constants obtained in this study are summarized in Table VI. Whereas the normal concentration of Glc-1,6-P_2 in brain is 100 μM (Passonneau et al., 1969), IMP appears

Table VI: Kinetic Constants of Glucose-bisphosphatase

reactant	parameter	value (μM)
Glc-1,6-P_2	K_m	20
mannose-1,6-P_2	K_m	20–39
IMP	K_a	9
GMP	K_a	70
Mg^{2+}	K_a	86
Glc-1-P	K_i	5[a]
Glc-6-P	K_i	18[a]

[a] Competitive with IMP.

to be undetectable (Schultz & Lowenstein, 1978), rendering the phosphatase essentially inactive. After electrical shock, IMP increased to a maximum of about 0.2 mM in less than 1 min; this was maintained briefly and then fell back to normal gradually. Passonneau et al. (1969) had observed that the half-time for the loss of Glc-1,6-P_2 in ischemic brain was less than 2 min. Both experimental states are associated with a rapid decrease in the ATP level. Therefore, it appears that the IMP-activated glucose-bisphosphatase may have a role in the maintenance of the ATP level in the brain. Glc-1,6-P_2 may inhibit a reaction that contributes to the control of ATP synthesis. When ATP falls, the rapid increase in IMP would activate glucose-bisphosphate phosphatase, releasing the inhibition. Although a number of enzymes related to carbohydrate metabolism have been shown to be affected by Glc-1,6-P_2 (see the introduction), the physiologically important interaction(s) is (are) yet to be identified.

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Registry No. IMP, 131-99-7; GMP, 85-32-5; Mg, 7439-95-4; Glc-1,6-P_2, 10139-18-1; mannose-1,6-P_2, 33112-59-3; Glc-1-P, 59-56-3; Glc-6-P, 56-73-5; ATP, 56-65-5; glucose diphosphatase, 69669-68-7.

References

Aldridge, W. N., & Thomas, M. (1966) *Biochem. J. 98*, 100–104.

Anderson, L., & Jolles, G. R. (1957) *Arch. Biochem. Biophys. 70*, 121–128.

Andrews, P. (1964) *Biochem. J. 91*, 222–233.

Beitner, R., & Nordenberg, J. (1979) *Biochim. Biophys. Acta 583*, 266–269.

Beitner, R., Haberman, S., & Livni, L. (1975) *Biochim. Biophys. Acta 397*, 355–369.

Berenblum, I., & Chain, E. (1938) *Biochem. J. 32*, 295–298.

Crane, R. K., & Sols, A. (1954) *J. Biol. Chem. 210*, 597–606.

Fleischer, S., & Kervina, M. (1974) *Methods Enzymol. 31A*, 6–18.

Gerber, G., Preissler, H., Heinrich, R., & Rapoport, S. M. (1974) *Eur. J. Biochem. 45*, 39–52.

Guha, S. K., & Rose, Z. B. (1982) *J. Biol. Chem. 257*, 6634–6637.

Gupta, R.K., & Benovic, J. L. (1978) *Biochem. Biophys. Res. Commun. 84*, 130–137.

Hashimoto, T., Joshi, J. G., Del Rio, C., & Handler, P. (1967) *J. Biol. Chem. 242*, 1671–1679.

Hofer, V. H. W., & Pette, D. (1968) *Hoppe-Seyler's Z. Physiol. Chem. 349*, 1378–1392.

Itaya, K., & Ui, M. (1966) *Clin. Chim. Acta 14*, 361–366.

Koster, J. F., Slee, R. G., Staal, G. E. J., & van Berkel, Th. J. C. (1972) *Biochim. Biophys. Acta 258*, 763–768.

Leloir, L. F., Trucco, R. E., Cardini, C. E., Paladini, A. C., & Caputto, R. (1948) *Arch. Biochem. Biophys. 19*, 339–340.

Lowry, O. H., & Passonneau, J. V. (1969) *J. Biol. Chem. 244*, 910–916.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Marcus, C. J. (1976) *J. Biol. Chem. 251*, 2963–2966.

Passonneau, J. V., Lowry, O. H., Schulz, D. W., & Brown, J. B. (1969) *J. Biol. Chem. 244*, 902–909.

Pasternak, T. (1953) *Helv. Chim. Acta 36*, 1614–1623.

Ray, W. J., Jr., & Roscelli, G. A. (1966) *J. Biol. Chem. 241*, 2596–2602.

Ray, W. J., Jr., & Peck, E. J., Jr. (1972) *Enzymes, 3rd Ed. 6*, 407–477.

Ray, W. J., Jr., Szymanki, E. S., & Ng, L. (1978) *Biochim. Biophys. Acta 522*, 434–442.

Rose, I. A., & Warms, J. V. B. (1974) *Biochem. Biophys. Res. Commun. 59*, 1333–1340.

Rose, I. A., Warms, J. V. B., & Kosow, D. P. (1974) *Arch. Biochem. Biophys. 164*, 729–735.

Rose, I. A., Warms, J. V. B., & Kaklij, G. (1975) *J. Biol. Chem. 250*, 3466–3470.

Rose, I. A., Warms, J. V. B., & Wong, L.-J. (1977) *J. Biol. Chem. 252*, 4262–4268.

Schultz, V., & Lowenstein, J. M. (1978) *J. Biol. Chem. 253*, 1938–1943.

Sillén, L. G., Martell, A. E., Högfeldt, E., & Smith, R. M. (1971) *Stability Constants*, Suppl. No. 1, p 642, The Chemical Society, London.

Toshima, Y., & Yoshimura, N. (1975) *J. Biochem. (Tokyo) 78*, 1161–1169.

Wimmer, M. J., & Rose, I. A. (1978) *Annu. Rev. Biochem. 47*, 1031–1078.

Wong, L.-J., & Rose, I. A. (1976) *J. Biol. Chem. 251*, 5431–5439.