amine, tertiary amine, oxygen, and thiol are not fluorescent in aqueous medium (unpublished results). Primary amines in DNA are at the N2 of guanine, N6 of adenine, and N4 of cytosine. The crystallogrphic data obtained with the iodoCpG–ellipticine complex (Jain et al., 1979) have indicated that when intercalated, the C10 of ellipticine can be very close to the primary amine of cytosine whereas the binding to the primary amine of either guanine or adenine should induce a strong modification in the arrangement of the DNA helix as postulated in the insertion–denaturation model. The binding process will be definitely determined by the identification of the chemical structure of nucleotide–NMHE adduct(s).

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Stereospecificity of the Fructose 2,6-Bisphosphate Site of Muscle 6-Phosphofructo-1-kinase†

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ABSTRACT: We explored the stereospecificity of the fructose 2,6-bisphosphate site of rabbit muscle 6-phosphofructo-1-kinase by determination of the activation constants (K_a) of several structurally locked analogues of this potent metabolic regulator. Under the assay conditions used, the K_a of fructose 2,6-bisphosphate was 0.12 μM. The most effective synthetic analogues and their K_a's were 2,5-anhydro-D-mannitol 1,6-bisphosphate (2.9 μM), 1,4-butanediol bisphosphate (6.6 μM), hexitol 1,6-bisphosphate (40 μM), and 2,5-anhydro-D-glucitol 1,6-bisphosphate (47 μM). Ten other bisphosphate compounds were much less effective as activators of the enzyme. These findings indicate that, unlike its active site, this allosteric site of 6-phosphofructo-1-kinase does not require the furanose ring. Its basic requirement seems to be a compound with two phosphate groups approximately 9 Å apart. Although the free hydroxy groups of the activator do not seem to be essential, their presence enhances appreciably the affinity of the ligand for this regulatory site.

The discovery of fructose 2,6-bisphosphate as an activator of 6-phosphofructo-1-kinase (Van Schaftingen et al., 1980a,b; Claus et al., 1981; Uyeda et al., 1981) opened new avenues of research on this enzyme. This modulator was discovered by workers investigating the effect of glucagon on hepatic 6-phosphofructo-1-kinase (Van Schaftingen et al., 1980a). The decrease in activity of the enzyme upon treatment of hepatocytes with glucagon was initially ascribed to be the result of its phosphorylation by cyclic AMP dependent protein kinase (Castano et al., 1979; Kagimoto & Uyeda, 1979, 1980; Claus et al., 1980). The potency of fructose 2,6-bisphosphate ac-

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tivation argues for a highly specific allosteric bisphosphate binding site (or sites).

Fructose 2,6-bisphosphate has been shown to be extremely acid-labile. Its hydrolysis produces equimolar amounts of fructose 6-phosphate and inorganic phosphate, with an accompanying decrease in ability to stimulate 6-phosphofructo-1-kinase activity (Van Schaftingen et al., 1980b). NMR analysis of the activator suggests that it has the β-anomeric configuration (Pilkis et al., 1981; Uyeda et al., 1981; Hesbain-Frisque et al., 1981). Fructose 2,6-bisphosphate relieves inhibition of 6-phosphofructo-1-kinase by ATP and increases enzyme affinity for fructose 6-phosphate. It is effective at submicromolar concentrations (Van Schaftingen et al., 1981). More recently Foe et al. (1983) showed that the much less effective activators of 6-phosphofructo-1-kinase, namely, fructose 1,6-bisphosphate and glucose 1,6-bisphosphate, bind also to the same regulatory site on the enzyme. Analogues of substrates, inhibitors, and activators have been utilized meaningfully as probes of active and regulatory sites of a number of enzymes (Hartman & Barker, 1965; Koerner et al., 1974, 1976; Fishbein et al., 1975; Wurster et al., 1976; Ogata et al., 1983). In this work, a number of fructose bisphosphate analogues were synthesized chemically and used to explore the structural specificity of the allosteric bisphosphate site(s) of 6-phosphofructo-1-kinase. Their activation constants (K_a) and ability to reverse citrate inhibition are reported.

Experimental Procedures

Materials. 6-Phosphofructo-1-kinase was isolated from rabbit muscle by the method of Ling et al. (1966) and stored as a stable preparation in 0.1 M potassium phosphate buffer (pH 8.0) containing 0.2 mM ethylenediaminetetraacetic acid (EDTA) and ammonium sulfate. Prior to use the enzyme was dialyzed against 0.1 M tris(hydroxymethyl)aminomethane (Tris)–PO_4 (pH 8.0) containing 0.2 mM EDTA to remove ammonium ions, which have been shown to activate 6-phosphofructo-1-kinase (Abrahams & Younathan, 1971). On the day of use, dialyzed 6-phosphofructo-1-kinase was diluted with a solution of 50 mM Tris–PO_4 (pH 8.0) containing 1 mM EDTA, 3 mM dithiothreitol, and 0.01% bovine serum albumin.

Rabbit muscle aldolase and α-glycerophosphate dehydrogenase–triosephosphate isomerase mixture were obtained from Sigma. These enzymes were dialyzed overnight against two changes of 0.25 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.8) containing 0.5 mM EDTA before use. ATP, reduced nicotinamide adenine dinucleotide (NADH), D-fructose 6-phosphate, D-fructose 1,6-bisphosphate, Tris, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), and methylenediphosphonic acid were all products of Sigma Chemical Co. All other chemicals were reagent grade.

1,2-Ethanediol bisphosphate, 1,3-propanediol bisphosphate, 1,4-butanediol bisphosphate, 1,5-pentanediol bisphosphate, 1,6-hexanediol bisphosphate, 1,7-heptanediol bisphosphate, 1,8-octanediol bisphosphate, and 1,9-nonanediol bisphosphate were synthesized by the method of Hartman and Barker (1965) and characterized by 50-MHz ^{13}C NMR spectroscopy. These compounds are more accurately named di-, tri-, tetramethylene, etc., bisphosphates. The analogues diethylene glycol bisphosphate and triethylene glycol bisphosphate were prepared in a similar manner. 2,5-Anhydro-D-glucitol 1,6-bisphosphate and 2,5-anhydro-D-mannitol 1,6-bisphosphate were prepared according to procedures we reported earlier (Voll et al., 1981). Fructose 2,6-bisphosphate was synthesized from fructose 1,6-bisphosphate by intramolecular cyclization followed by alkaline hydrolysis as described by Van Schaftingen and Hers (1981). Its concentration was determined enzymatically. The sample to be analyzed was treated with 1 N HCl for 30 min, neutralized with NaOH, and assayed for fructose 6-phosphate by using the method of Zalitis and Oliver (1967).

Hexitol 1,6-bisphosphate was synthesized by the borohydride reduction of fructose 1,6-bisphosphate (Ginsburg & Mehler, 1966). An enzymatic procedure was utilized to remove a small amount of unreacted fructose 1,6-bisphosphate from the mixture. A sample of the hexitol 1,6-bisphosphate mixture was reacted with excess aldolase for 24 h at 28 °C and then applied to a Dowex 1-X8 ion-exchange column (Cl^- form). The column was developed with a NaCl gradient (0.1–0.4 M). The eluant was assayed for acid-labile phosphate (Cooper, 1977) and 6-phosphofructo-1-kinase activating ability. Column fractions that showed 6-phosphofructo-1-kinase activating ability exhibited no activity with aldolase. Pooled fractions were treated with Dowex 50 (H^+ form). Cyclohexylamine was added to the resulting acidic filtrate (pH 2) until pH 12 was reached. The solution was concentrated under vacuum to a solid, which was characterized as an equimolar mixture of two hexitol 1,6-bisphosphates, namely, glucitol 1,6-bisphosphate and mannitol 1,6-bisphosphate, by ^{13}C NMR spectroscopy.

Enzymatic Assays. For evaluation of 6-phosphofructo-1-kinase activating ability, a system that contained an inhibitory level of ATP at pH 7.2 was employed. Each cuvette contained, in a total volume of 1.0 mL, 50 mM Hepes buffer (pH 7.2), 50 mM KCl, 4.0 mM $MgCl_2$, 0.2 mM EDTA, 0.16 mM NADH, 1.0 mM ATP, 0.25 mM fructose 6-phosphate, 1.0 mM dithiothreitol, 3.33 units of aldolase, 5.8 units of α-glycerophosphate dehydrogenase–triosephosphate isomerase mixture, approximately 0.7 μg of 6-phosphofructo-1-kinase, and a 1.0 mM concentration of the compound to be tested. The amount of auxiliary coupling enzymes present was in large excess to avoid being rate-limiting and to prevent loss of aldolase activity, as some of the bisphosphate analogues have been shown to be mild inhibitors of aldolase (Hartman & Barker, 1965). The reaction was initiated with fructose 6-phosphate. Conditions were chosen such that the velocity of the reaction in the absence of any activating substance was essentially zero. Compounds that did not show 6-phosphofructo-1-kinase activation were tested at levels up to 5.0 mM.

The system used to determine activation constants was essentially that given above with the exception that the concentration of activator was varied. The activation constant (K_a; Atkinson, 1966) was defined as that concentration of modifier which gives half-maximal activation at a specified substrate concentration. The substrate concentration used in all assays was 0.25 mM fructose 6-phosphate.

Enzymatic activity was measured as the decrease in absorbance of NADH at 340 nm on a Gilford Model 250 recording spectrophotometer at 28 °C. Activation constants and V_{max} were estimated by using the HYPERBOLIC program in the PROPHET system (Johnson, 1979), which is based on a weighted least-squares analysis of the data (Cleland, 1967).

Results

Of the analogues tested for their ability to activate ATP-inhibited 6-phosphofructo-1-kinase, nine showed such ability to varying degrees. Structures of some of the compounds found to be activators of 6-phosphofructo-1-kinase are shown in Figure 1, and their activation constants (at 0.25 mM fructose 6-phosphate) are given in Table I. Compounds that exhibited activation in the range observed with fructose 2,6-bisphosphate (0.12 μM) and their respective activation constants were

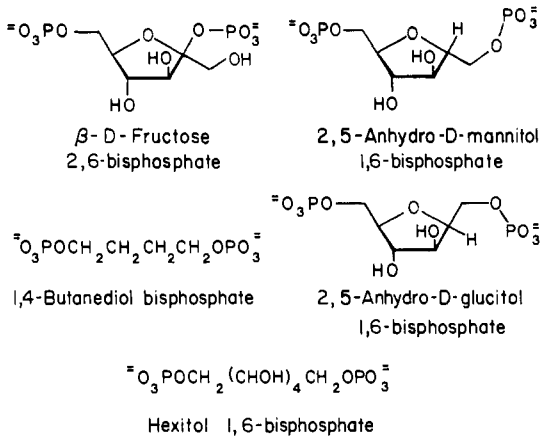

FIGURE 1: Structures of some of the bisphosphates shown to be activators of 6-phosphofructo-1-kinase.

Table I: Compounds Tested as Activators of ATP-Inhibited 6-Phosphofructo-1-kinase

compd	K_a (M)	distance[a] (Å) max	min
fructose 2,6-bisphosphate	$(1.2 \pm 0.2) \times 10^{-7}$	9.0	5.9
methylenediphosphonate	no activation	2.7	2.7
1,2-ethanediol bisphosphate	$>3.5 \times 10^{-3}$ [b]	7.0	5.9
1,3-propanediol bisphosphate	$(2.7 \pm 1.1) \times 10^{-4}$	8.2	5.9
1,4-butanediol bisphosphate	$(6.6 \pm 0.8) \times 10^{-6}$	9.4	5.9
2,5-anhydro-D-glucitol 1,6-bisphosphate	$(4.7 \pm 0.8) \times 10^{-5}$	10.0	5.9
2,5-anhydro-D-mannitol 1,6-bisphosphate	$(2.9 \pm 0.3) \times 10^{-6}$	10.2	5.9
1,5-pentanediol bisphosphate	$(1.1 \pm 0.3) \times 10^{-3}$	10.6	5.9
diethylene glycol bisphosphate	$(5.0 \pm 1.8) \times 10^{-4}$	10.6	5.9
1,6-hexanediol bisphosphate	$>4.0 \times 10^{-3}$ [b]	11.8	5.9
hexitol 1,6-bisphosphate	$(4.0 \pm 0.9) \times 10^{-5}$	11.8	5.9
1,7-heptanediol bisphosphate	no activation	13.0	5.9
1,8-octanediol bisphosphate	no activation	14.2	5.9
triethylene glycol bisphosphate	no activation	14.2	5.9
1,9-nonanediol bisphosphate	no activation	15.4	5.9

[a] Maximal distance between phosphorus atoms measured on Drieding models. Minimal distance based on the P–O bond length and the van der Waals radii of the two oxygen atoms. Data similar to that of Hartman and Barker (1965). [b] Value estimated from visual interpretation of double reciprocal plots.

2,5-anhydro-D-mannitol 1,6-bisphosphate (2.9 μM), 1,4-butanediol bisphosphate (6.6 μM), hexitol 1,6-bisphosphate (40 μM), and 2,5-anhydro-D-glucitol 1,6-bisphosphate (47 μM). The constants of other bisphosphate analogues that showed activation were well in excess of these values (Table I). These data and an examination of the maximal phosphate–phosphate distances, as measured on Drieding models, suggest that the allosteric bisphosphate binding site of 6-phosphofructo-1-kinase requires that the distance between the two phosphate groups of the activator be in the range of 9.0–10.2 Å.

The effect of fructose 2,6-bisphosphate and 1,4-butanediol bisphosphate on citrate inhibition of 6-phosphofructo-1-kinase is illustrated in Figure 2. Citrate is one of the physiological inhibitors of 6-phosphofructo-1-kinase. Both fructose 2,6-bisphosphate and 1,4-butanediol bisphosphate reversed this inhibition to a great extent (Figure 2). Citrate at 10 μM brought about more than 50% inhibition of 6-phosphofructo-1-kinase activity in the absence of bisphosphate compounds. In the presence of fructose 2,6-bisphosphate (1.5 and 15 nM), 70% and 85%, respectively, of the initial activity remained at 10 μM citrate. Incorporation of 50 μM 1,4-butanediol bisphosphate in the assay medium resulted in the retention of 85% of the initial activity at the same level of citrate. These data

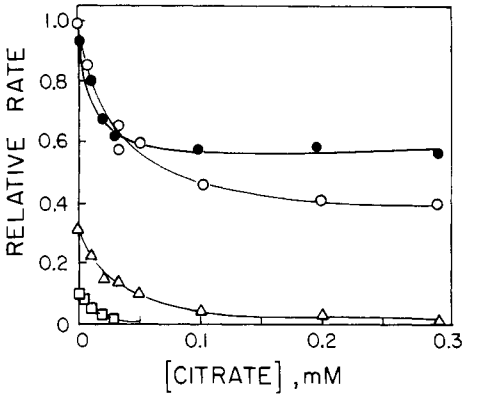

FIGURE 2: Reversal of inhibitory effect of citrate on 6-phosphofructo-1-kinase by fructose 2,6-bisphosphate and 1,4-butanediol bisphosphate. Conditions used: ATP, 0.5 mM; fructose 6-phosphate, 0.5 mM; and 6-phosphofructo-1-kinase, 0.7 μg per 1.0 mL of assay mixture. The specific activity at the 100% point is 40 units per milligram of protein. Other conditions are as explained under Experimental Procedures. Symbols: (□) no activator, (△) 1.5 nM fructose 2,6-bisphosphate, (○) 15 nM fructose 2,6-bisphosphate, and (●) 50 μM 1,4-butanediol bisphosphate.

indicate that binding of fructose 2,6-bisphosphate or 1,4-butanediol bisphosphate at the bisphosphate binding site stabilizes the enzyme in an active conformation which is more resistant to inhibition by citrate.

Discussion

Among the 15 bisphosphates used in this study to explore the specificity of this allosteric site of 6-phosphofructo-1-kinase, fructose 2,6-bisphosphate was found to be the most potent modulator. It was 24 times more effective in activating 6-phosphofructo-1-kinase than the next closest activator 2,5-anhydro-D-mannitol 1,6-bisphosphate, 55 times more effective than 1,4-butanediol bisphosphate, and 390 times more effective than 2,5-anhydro-D-glucitol 1,6-bisphosphate. Whereas the maximal phosphate–phosphate distance in 2,5-anhydro-D-glucitol 1,6-bisphosphate (10.0 Å) is closer to that of fructose 2,6-bisphosphate (9.0 Å), 2,5-anhydro-D-mannitol 1,6-bisphosphate with a slightly longer maximal distance (10.2 Å) showed much greater activation than 2,5-anhydro-D-glucitol 1,6-bisphosphate. This result can be explained by the ability of the C-1 phosphate group of 2,5-anhydro-D-mannitol 1,6-bisphosphate to mimic sterically the C-2 phosphate group of the β-anomer of fructose 2,6-bisphosphate by rotation about the O-1–C-1 and C-1–C-2 bonds (Figure 1). Such rotation is sterically allowed because 2,5-anhydro-D-mannitol 1,6-bisphosphate does not have a hydroxy group on C-2 (such a hydroxy group occurs on β-fructofuranose 1,6-bisphosphate). Similar rotation does not bring about such a favorable isosteric structure in the case of 2,5-anhydro-D-glucitol 1,6-bisphosphate. Undoubtedly, the limited allowed conformational change in the activating analogues is influenced by the structure of the regulatory site of the enzyme, and this might differ somewhat according to the species from which it is obtained.

The potency of the activation shown by 1,4-butanediol bisphosphate was surprising. This compound approximates the atomic spacing across the C-2 to C-6 part of the fructose 2,6-bisphosphate molecule. The number of atoms is identical and differs only in the substitution in 1,4-butanediol bisphosphate of a methylene group for the ring oxygen in fructose 2,6-bisphosphate. Due to repulsion between phosphate groups, 1,4-butanediol bisphosphate (and other linear bisphosphate analogues) exist as the zigzag conformation in solution (Hartman & Barker, 1965). The potency of this open chain

compound indicates that the furanose ring is not essential for activation. In this regard, the allosteric site of 6-phosphofructo-1-kinase is quite different from its active site, which we have shown to require the furanose ring for activity (Koerner et al., 1974; Younathan et al., 1981). Recently, Poorman et al. (1984) speculated that the fructose bisphosphate regulatory site of rabbit muscle 6-phosphofructo-1-kinase is a mutation of its active site.

The presence of hydroxyls is obviously not a major requirement for binding to the bisphosphate site of 6-phosphofructo-1-kinase, as indicated by the activity of 1,4-butanediol bisphosphate. However, hydroxy groups probably play a role in the binding as exhibited by hexitol 1,6-bisphosphate, an open chain analogue with hydroxyls on C-2 through C-5. This compound is 100-fold more effective than hexanediol 1,6-bisphosphate even though both compounds have the same maximal phosphate–phosphate distance (11.8 Å). Interaction of hydroxyls at a specific binding site (or sites) on the enzyme could decrease the phosphate–phosphate distance, thereby increasing the affinity for binding. Another possibility that could explain the effectiveness of hexitol 1,6-bisphosphate as an activator is that dipole interactions between the hydroxyls could cause the carbon chain to assume a conformation (and therefore phosphate–phosphate separation) similar to that of the furanose ring system (Hartman & Barker, 1965).

Finally, the present data corroborate our explanation of the mechanism of the hypoglycemic action of 2,5-anhydro-D-mannitol (Hanson et al., 1984). Riquelme et al. (1984) reported that 2,5-anhydro-D-mannitol was phosphorylated to the 1-phosphate and the 1,6-bisphosphate esters in rat hepatocytes. We have shown that the latter compound stimulates glycolysis and simultaneously inhibits both gluconeogenesis and glycogenolysis in the same system (Hanson et al., 1984).

Registry No. $H_2PO_4(CH_2)_2O_4PH_2$, 84351-11-1; $H_2PO_4(CH_2)_3O_4PH_2$, 674-67-9; $H_2PO_4(CH_2)_4O_4PH_2$, 763-26-8; $H_2PO_4(CH_2)_5O_4PH_2$, 763-27-9; $H_2PO_4(CH_2)_6O_4PH_2$, 674-70-4; $H_2PO_4(CH_2)_7O_4PH_2$, 100113-38-0; $H_2PO_4(CH_2)_8O_4PH_2$, 674-71-5; $H_2PO_4(CH_2)_9O_4PH_2$, 100113-39-1; fructose 2,6-bisphosphate, 77164-51-3; methylenediphosphonate, 1984-15-2; 2,5-anhydro-D-glucitol 1,6-bisphosphate, 4429-47-4; diethylene glycol bisphosphate, 45163-42-6; hexitol 1,6-bisphosphate, 17230-91-0; triethylene glycol bisphosphate, 52329-58-5; 6-phosphofructo-1-kinase, 9001-80-3; 2,5-anhydro-D-mannitol 1,6-bisphosphate, 671-08-9.

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