Binding of Hexose Bisphosphates to Muscle Phosphofructokinase†

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ABSTRACT: On the basis of kinetic activation assays, the apparent affinity of muscle phosphofructokinase for fructose 2,6-bisphosphate was about 9-fold greater than that for fructose 1,6-bisphosphate, which in turn was about 10 times higher than that for glucose 1,6-bisphosphate. Equilibrium binding experiments showed that both fructose bisphosphates bind to phosphofructokinase with negative cooperativity; the affinity for fructose 2,6-bisphosphate was about 1 order of magnitude greater than the affinity for fructose 1,6-bisphosphate. Binding of fructose 2,6-bisphosphate to phosphofructokinase was antagonized by fructose 1,6-bisphosphate and glucose 1,6-bisphosphate and vice versa. Both fructose bisphosphates promoted aggregation of the enzyme to higher polymers as indicated by sucrose density gradient centrifugation. Other indicators of phosphofructokinase conformation such as thiol reactivity and maximum activation of in vitro phosphorylation by the catalytic subunit of cyclic AMP-dependent protein kinase gave identical results in the presence of fructose 2,6-bisphosphate, fructose 1,6-bisphosphate, or glucose 1,6-bisphosphate, indicating a common conformation is produced by all three ligands. It is concluded that the sugar bisphosphates bind to a single site on the enzyme.

Fructose 2,6-bisphosphate (fructose-2,6-P_2)[1] is a potent (Hers et al., 1982; Hers & Van Schaftingen, 1982; Uyeda et al., 1982; Pilkis et al., 1982a) activator of phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11). This compound was originally discovered in and isolated from liver extracts, where it was shown to play a key role in the regulation of hepatic glycolysis and gluconeogenesis. Further research showed that fructose-2,6-P_2 was also present in many other tissues and, in fact, could be found in such diverse organisms as yeasts and plants (Hers et al., 1982; Hers & Van Schaftingen, 1982). It was postulated that fructose-2,6-P_2 could be regarded in a general sense as a metabolic signal, signifying the presence of glucose, in contrast to cyclic AMP, which generally signifies the absence of glucose (Hers & Van Schaftingen, 1982).

The site of action of fructose-2,6-P_2 on phosphofructokinase has not yet been determined. It might be presumed that it acts at the same site on phosphofructokinase as the well-known activator fructose-1,6-P_2; the results of some kinetic experiments using erythrocyte phosphofructokinase are consistent with this idea (Heylen et al., 1982). However, fructose-2,6-P_2 was reported to activate liver phosphofructokinase at concentrations 3 orders of magnitude lower than concentrations of fructose-1,6-P_2 required for the same effect (Van Schaftingen et al., 1981). Also phosphofructokinase from Ehrlich ascites tumor cells was reported to be activated by micromolar concentrations of fructose-2,6-P_2 but was not activated at all by up to 2 mM fructose-1,6-P_2 (Bosca et al., 1982). Therefore, the possibility was raised that fructose-2,6-P_2 might act at a site on phosphofructokinase distinct from the fructose-1,6-P_2 site. In the present study, we examine this question.

Experimental Procedures

Enzymes and Substrates. Phosphofructokinase was purified from fresh rabbit muscle as described by Kemp (1975) and quantitated by the method of Bradford (1976) as described previously (Foe & Kemp, 1982) or by absorbance at 279 nm (Parmeggiani et al., 1966). When necessary, traces of aldolase were removed by passage over Sepharose CL-4B or Sephacryl S-300. Auxiliary enzymes for kinetic assays were purchased from Sigma. The catalytic subunit of cyclic AMP dependent protein kinase was prepared by the method of Sugden et al. (1976). ATP, NADH, NADP, glucose-1-P, fructose-6-P, fructose-1,6-P_2, and glucose-1,6-P_2 were obtained from Sigma or Boehringer. Stock solutions of ATP were adjusted to pH 7.0 with NaOH and standardized by measuring absorbance at 259 nm. The ATP stock solution typically contained about 1% ADP as determined by enzymatic assay with pyruvate kinase and lactate dehydrogenase. Fructose-6-P solutions were standardized by enzymatic assay, either by coupling to reduction of NADP via phosphoglucosoisomerase and glucose-6-phosphate dehydrogenase or by coupling to oxidation of NADH via phosphofructokinase, aldolase, triosephosphate isomerase, and glycerolphosphate dehydrogenase. Glucose-1,6-P_2 was standardized by enzymatic assay of glucose-6-P formed after hydrolysis in 1 N HCl at 100 °C for 10 min. Glucose-1,6-P_2 stocks were also assayed for fructose-1,6-P_2; none was found. Fructose-2,6-P_2 was synthesized as described previously (Gottschalk et al., 1982) or was purchased from Sigma. Except when used in kinetic assays, fructose-2,6-P_2 was further purified by heating in 0.25 N NaOH at 95 °C for 20 min followed by neutralization and passage over Sephadex G-15. The small amounts of fructose-6-P and fructose-1,6-P_2 present in fructose-2,6-P_2 preparations prior to this treatment had no effect on phosphofructokinase kinetic assays. The concentration of fructose-2,6-P_2 was determined by enzymatic assay of fructose-6-P before and after hydrolysis in 0.1 N HCl for 20 min.

Radioactive Ligands. [γ-^{32}P]ATP, [8-^{3}H]cAMP, and [U-^{14}C]fructose-6-P were obtained from Amersham. Fructose-2,6-[2-^{32}P]P_2 was prepared as follows. Fructose-6-P 2-kinase was purified from a single rat liver according to Furuya et al. (1982), except that after ion-exchange chromatography, the preparation was precipitated with ammonium sulfate and subjected to gel filtration on Sephacryl S-200 instead of

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[1] Abbreviations: fructose-2,6-P_2, fructose 2,6-bisphosphate; fructose-6-P, fructose 6-phosphate; fructose-1,6-P_2, fructose 1,6-bisphosphate; glucose-1,6-P_2, glucose 1,6-bisphosphate; Tes, 2-[[tris(hydroxymethyl)-methyl]amino]ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; LDH, lactate dehydrogenase.

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Sephadex G-200. A 2-mL portion (about 2 milliunits) of the purified enzyme in 50 mM Tris–phosphate, 0.2 mM EGTA, 1 mM dithiothreitol, and 0.1 M NaCl pH 8.0, was incubated in the presence of 10 mM $MgCl_2$, 5 mM fructose-6-P, and 2.5 mM [γ-^{32}P]ATP (140 cpm/pmol) for 5.5 h at 30 °C. The reaction solution was washed twice with 40 mg of Norit, and 0.05 volume of 5 N NaOH was added. The solution was incubated in a boiling H_2O bath for 20 min, cooled, and adjusted to pH 9 with 1 N acetic acid. The solution was lyophilized, redissolved in 600 μL of 0.1 M ammonium bicarbonate, and applied to a 1 × 60 cm Sephadex G-15 column equilibrated with the same buffer. The initial radioactive peak, which coincided with phosphofructokinase-activating activity, was applied to a 1.5 × 6 cm column of DE-52 equilibrated with 0.1 M ammonium bicarbonate and eluted with a linear 0.1–0.3 M gradient of the same buffer (total volume 100 mL). The major peak of radioactivity, coinciding with phosphofructokinase-activating activity, was pooled, lyophilized, redissolved in 600 μL of 0.1 M ammonium bicarbonate, and stored frozen at −20 °C. The radioactive fructose-2,6-P_2 appeared to be pure by the following criteria. First, its specific radioactivity was approximately equal to that of the original [γ-^{32}P]ATP used in its synthesis. Second, when incubated with 0.09 N HCl for 60 min and reapplied to the Sephadex G-15 column, 95% of the applied radioactivity coeluted with inorganic phosphate.

Fructose-1,6-[1-^{32}P]P_2 was synthesized as follows. To 1 mL of solution containing 0.1 M ammonium bicarbonate, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 1 mM [γ-^{32}P]ATP, and 1 mM fructose-6-P, pH 8.0, was added 3 μg of phosphofructokinase. The solution was incubated at room temperature for 40 min. After being heated in a boiling water bath for 3 min, the solution was applied to a 1 × 15 cm column of DEAE-cellulose (Whatman, DE-52) equilibrated with 0.1 M ammonium bicarbonate. The column was washed with the same buffer and eluted with a 0.1–0.3 M ammonium bicarbonate gradient, 100-mL total volume. The fructose-1,6-P_2 peak was lyophilized, redissolved in 0.1 M ammonium bicarbonate, and rechromatographed on DEAE-cellulose as described above. The radioactive peak fractions were pooled, lyophilized, redissolved in water, and stored frozen. The preparation was free of fructose-6-P and adenine nucleotides.

Glucose-1,6-[6-^{32}P]P_2 was synthesized as follows. To 1.15 mL of a solution containing 5 mM Tris–SO_4, 4.3 mM [γ-^{32}P]ATP, 5.1 mM glucose-1-P, 9 mM $MgCl_2$, 1 mM EDTA, and 2 mM dithiothreitol, pH 8.0, was added 325 μg of phosphofructokinase. The solution was incubated overnight at room temperature and then made 0.25 N in NaOH and placed in a boiling water bath for 25 min. After neutralization with acetic acid, the solution was applied to a 1 × 60 cm column of Sephadex G-15 equilibrated with 0.1 M ammonium bicarbonate. Fractions corresponding to the large radioactive peak areas were pooled and applied to a 1 × 24 cm column of DEAE-cellulose, which was washed and eluted as described above for fructose-1,6-P_2. The radioactive peak was lyophilized, redissolved in water, and stored frozen. The preparation contained no glucose-6-P, fructose-6-P, fructose-1,6-P_2, or adenine nucleotides.

Phosphofructokinase Assay Techniques. All of the following assays were performed at 30 °C. Assay A consisted of 50 mM Na/Tes, pH 7.0, containing 150 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 0.2 mM NADH, 0.2 mM phosphoenolpyruvate, 1 mM ATP, 0.5 mM fructose-6-P, and the following concentrations of desalted auxiliary enzymes: 40 μg/mL aldolase, 10 μg/mL glycerolphosphate dehydrogenase, 10 μg/mL pyruvate kinase, and 2 μg/mL triosephosphate isomerase. The reaction was initiated by adding fructose-6-P to the otherwise complete assay solution, to which 10–20 milliunits/mL phosphofructokinase and the indicated concentration of sugar bisphosphate had already been added. After a 1–2-min lag period, the linear decrease in absorbance at 340 nm was measured.

Assay B had the same composition as assay A except the NADH was 80 μM, the fructose-6-P was 1 mM, and the concentrations of desalted auxiliary enzymes were as follows; 20 μg/mL pyruvate kinase, 20 μg/mL lactate dehydrogenase, and 50 μg/mL aldolase. When fructose-1,6-P_2 was added to the assay solution, its concentration was calculated according to the equilibrium constant for the aldolase reaction of 45 μM (Tornheim & Lowenstein, 1976).

Assay C had the same composition as assay A except that NADH, phosphoenolpyruvate, and the auxiliary enzymes were omitted. In addition, the concentration of ATP was 0.1 mM, and the concentration of [U-^{14}C]fructose-6-P was 20 μM. The reaction was initiated by adding phosphofructokinase (0.5–1 milliunit/mL) to the complete assay system, including an indicated concentration of sugar bisphosphate. The rate of reaction was determined as follows. At times of 0, 1, and 2 min, 100-μL aliquots of reaction solution were added to 400 μL of 1 M formic acid/ammonium formate, pH 3. The acidified samples were then applied to small (0.5-mL) columns of Dowex AG 1-X4 equilibrated with the same formic acid/ammonium formate buffer. Unreacted fructose-6-P was eluted by washing the columns with 5 mL of the same buffer. Radioactive fructose-1,6-P_2 was then eluted with 1.5 mL of 4 M formic acid/ammonium formate, pH 3, and collected in scintillation vials. The contents of the vials were then counted for radioactivity.

Sucrose Gradients. Phosphofructokinase was equilibrated against 50 mM Na/Tes, pH 7.2, containing 1 mM EDTA, 0.1 mM ATP, and 1 mM dithiothreitol by passage through a column of Sephadex G-50 fine. The enzyme was diluted to 1.0 mg/mL in the same buffer, and 100-μL aliquots were applied to 5–20% sucrose gradients in the same buffer, with additions as indicated. The gradients were centrifuged at 39 000 rpm in an SW50.1 rotor at 4 °C for 6 h. Pyruvate kinase (s_{20} = 10.0 S), centrifuged in a separate gradient, was used as a standard.

Thiol-Group Titrations. Phosphofructokinase was equilibrated with 25 mM sodium β-glycerophosphate/25 mM glycylglycine, and 1 mM EDTA, pH 8.5, by desalting over Sephadex G-50 fine. The enzyme was diluted to 0.2 mg/mL with the same buffer and placed in a cuvette. Effectors were added as indicated, followed by addition of DTNB. The change in absorbance at 412 nm was immediately monitored at 30 °C. In experiments carried out at pH 7.0, the procedure was as above except the buffer also contained 6 mM $MgCl_2$.

Phosphorylation of Phosphofructokinase. Phosphofructokinase (1.8 mg/mL) was phosphorylated in a buffer consisting of 50 mM Na/Tes, pH 7.2, containing 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.58 mM [γ-^{32}P]ATP (100–200 cpm/pmol), and effector as indicated. The reaction was initiated with the addition of the catalytic subunit of cAMP-dependent protein kinase to a final concentration of 5 μg/mL. Aliquots were withdrawn at 5 and 10 min, spotted onto filter paper squares that were subsequently washed in cold 10% trichloroacetic acid, and counted for ^{32}P as described by Corbin & Reimann (1974). The reaction course was linear at these time points. Because commercial preparations of ATP contain ADP as a minor contaminant, equilibration of ^{32}P label oc-

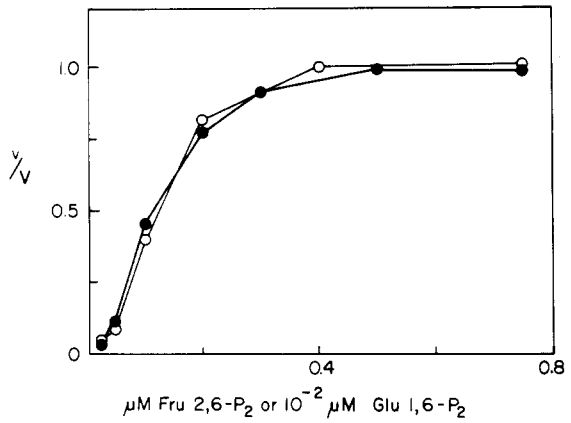

FIGURE 1: Activation of phosphofructokinase by Fru-2,6-P_2 (●) and Glu-1,6-P_2 (○). Assay A was used. In this and in following figures, Fru-2,6-P_2, Fru-1,6-P_2 and Glu-1,6-P_2 signify fructose-2,6-P_2, fructose-1,6-P_2, and glucose-1,6-P_2, respectively.

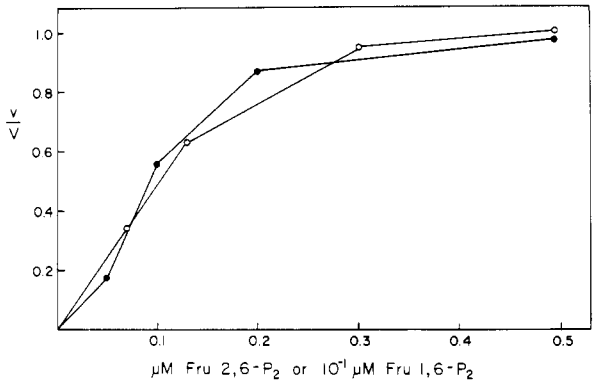

FIGURE 2: Activation of phosphofructokinase by Fru-2,6-P_2 (○) and Fru-1,6-P_2 (●). Assay B was used.

curred between ATP and fructose-1,6-P_2 when fructose-1,6-P_2 was present in the reaction solution. The effect was to lower the specific activity of [γ-^{32}P]ATP: this effect was corrected for when calculating incorporation of ^{32}P into phosphofructokinase in the presence of fructose-1,6-P_2.

Equilibrium Binding Experiments. These were performed by the gel-filtration technique (Hummel & Dreyer, 1962) according to Kemp & Krebs (1967). Phosphofructokinase (50–1500 μg in 1.5 mL) was applied to a 1.5 × 24 cm column of Sephadex G-50 fine equilibrated with 25 mM glycylglycine, 25 mM glycerol β-phosphate, 1 mM EDTA, and 5 mM mercaptoethanol, all at pH 6.95, plus the indicated concentration of radiolabeled ligand. The column was eluted at room temperature at a flow rate of 4–5 mL/h, and fractions of 1.5 mL were collected.

Results

Comparative Activation of Phosphofructokinase by Sugar Bisphosphates. Figure 1 shows activation of phosphofructokinase by fructose-2,6-P_2 and glucose-1,6-P_2 in the aldolase-coupled spectrophotometric assay (assay A). The concentration of fructose-2,6-P_2 necessary for half-maximal activation was 0.11 μM, while that for glucose-1,6-P_2 was 12.5 μM. In comparison, when activation by fructose-2,6-P_2 and fructose-1,6-P_2 was compared by the aldolase-buffered pyruvate kinase–lactate dehydrogenase coupled assay (assay B), half-activation was reached at about 0.1 μM for fructose-2,6-P_2 and at about 0.9 μM for fructose-1,6-P_2 (Figure 2). Therefore, fructose-2,6-P_2 was about an order of magnitude more potent than fructose-1,6-P_2 as an activator of phosphofructokinase, which in turn was about an order of magnitude more potent than glucose-1,6-P_2. This overall picture was confirmed by the radioactive assay (assay C). Here, half-activation concentrations of 0.15, 1.3, and 7.5 μM were obtained for fructose-2,6-P_2, fructose-1,6-P_2, and glucose-1,6-P_2, respectively (Figure 3). In this case, the substrate concentrations were much lower than in the spectrophotometric assays because of the need to obtain low blanks, but similar ratios of half-activation concentrations of sugar bisphosphates were still observed. That the absolute half-activation concentrations were similar to those seen in the spectrometric assays was probably a fortuitous result due to the low concentration of ATP compensating for the low concentration of fructose-6-P in the radioactive assay.

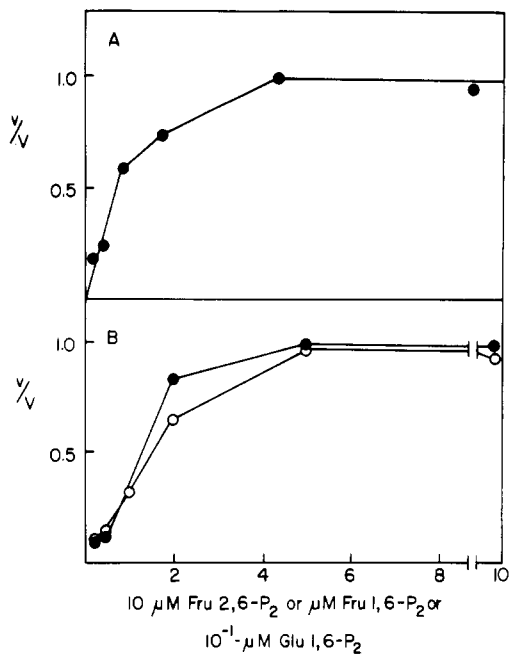

FIGURE 3: Activation of phosphofructokinase by sugar bisphosphates: (A) activation by Glu-1,6-P_2; (B) activation by Fru-1,6-P_2 (●) and Fru-2,6-P_2 (○). Assay C was used.

Binding of Fructose Bisphosphates to Phosphofructokinase. Both fructose-2,6-P_2 and fructose-1,6-P_2 bound to phosphofructokinase with negative cooperativity (Figure 4). This is in contrast to adenine nucleotide activators, fructose-6-P, and the inhibitor citrate, which bind to phosphofructokinase non-cooperatively under the same conditions (Kemp & Krebs, 1967; Colombo et al., 1975). Both Scatchard plots bent sharply at around 0.5 mol of ligand bound per mol of phosphofructokinase subunit (Figure 4). This may indicate that the binding of either fructose bisphosphate to two subunits per phosphofructokinase tetramer greatly reduces the affinity for binding to the other two subunits. Because the enzyme is composed of identical subunits and because a binding stoichiometry of 1 mol of fructose bisphosphate/mol of subunit is indicated, the existence of two types of binding sites is an unlikely explanation for the observed curvature of the Scatchard plots. Half-stoichiometric binding of fructose-2,6-P_2 occurs at a concentration of about 0.07 μM, while half-stoichiometric binding of fructose-1,6-P_2 occurs at about 1 μM. Thus, in agreement with the kinetic data, the affinity of fructose-2,6-P_2 for phosphofructokinase appears to be about 1 order of magnitude greater than the affinity of fructose-1,6-P_2 for the enzyme.

Table I shows that each fructose bisphosphate inhibited the binding of the other to phosphofructokinase; furthermore, each fructose bisphosphate enhanced the binding of cyclic AMP and inhibited the binding of MgATP. In addition, glucose-

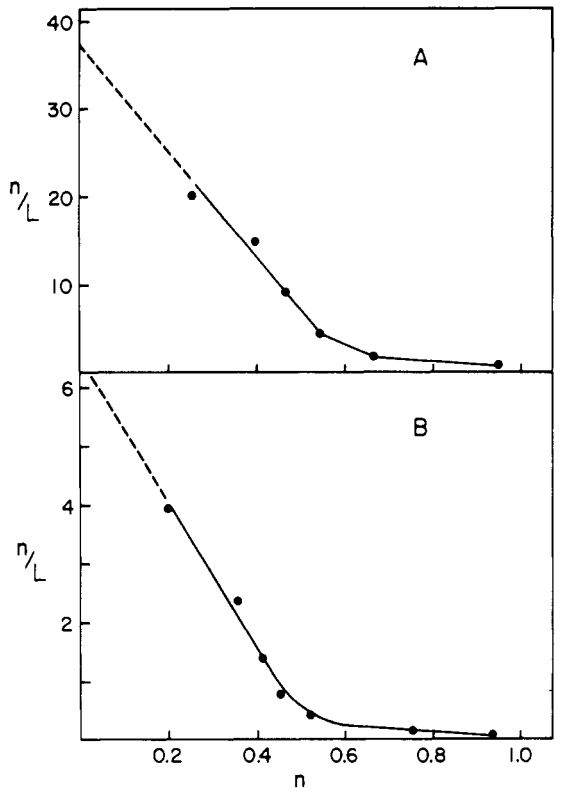

FIGURE 4: Binding of fructose bisphosphates to phosphofructokinase: (A) binding of Fru-2,6-P_2; (B) binding of Fru-1,6-P_2. n is defined as the number of moles of ligand bound per mole of phosphofructokinase subunit (M_r 84 000); L is the concentration of ligand in units of 10^{-6} M.

Table I: Equilibrium Binding of Ligands by Phosphofructokinase

radioactive ligand (μM)	n	n (competing ligand)
Fru-1,6-P_2 (0.2)	0.27	0.006 (2 μM Fru-2,6-P_2)
Fru-1,6-P_2 (1.2)	0.52	0.36 (20 μM Glu-1,6-P_2)
Fru-2,6-P_2 (0.1)	0.50	0.19 (2 μM Fru-1,6-P_2)
Fru-2,6-P_2 (0.05)	0.47	0.15 (10 μM Glu-1,6-P_2)
Glu-1,6-P_2 (5)	0.16	0.01 (2 μM Fru-2,6-P_2)
Glu-1,6-P_2 (5)	0.16	0.05 (2 μM Fru-1,6-P_2)
cyclic AMP (0.2)	0.14	0.28 (2 μM Fru-2,6-P_2)
cyclic AMP (0.2)	0.14	0.18 (2 μM Fru-1,6-P_2)
MgATP (1)	0.22	0.11 (2 μM Fru-2,6-P_2)
MgATP (1)	0.22	0.17 (2 μM Fru-1,6-P_2)

[a] See Experimental Procedures for conditions. The quantity n is defined as the number of moles of radioactive ligand bound per mole of enzyme subunit, M_r 84 000, at pH 6.95, in glycylglycine (25 mM), glycerophosphate (25 mM), EDTA (1 mM), mercaptoethanol (5 mM), and the indicated ligand and competing ligand concentration.

1,6-P_2 inhibited the binding of fructose-2,6-P_2 and fructose-1,6-P_2 and vice versa. These data are consistent with the idea that all three sugar bisphosphates bind to a common site.

Self-Association of Phosphofructokinase in the Presence of Sugar Bisphosphates. Figure 5 shows the sedimentation of phosphofructokinase centrifuged in the presence of 0.1 mM AMP, 0.1 mM fructose-1,6-P_2, or 30 μM fructose-2,6-P_2. These are all essentially saturating concentrations of each ligand. It is obvious that the sedimentation behavior of phosphofructokinase in the presence of fructose-2,6-P_2 is similar to that observed in the presence of fructose-1,6-P_2 and dissimilar to that observed in the presence of AMP. Although the phosphofructokinase peaks were not sharp, the sedimentation coefficient of phosphofructokinase in the presence of either fructose bisphosphate could be estimated at about 21 S compared with a value of 18 S in the presence of AMP. In the absence of added activators, the sedimentation coefficient was about 19 S (not shown).

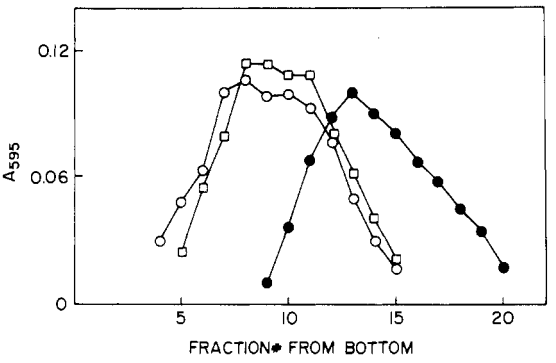

FIGURE 5: Sedimentation of phosphofructokinase in the presence of activators. See text for conditions. (○) Plus 30 μM Fru-2,6-P_2; (□) plus 100 μM Fru-1,6-P_2; (●) plus 100 μM AMP.

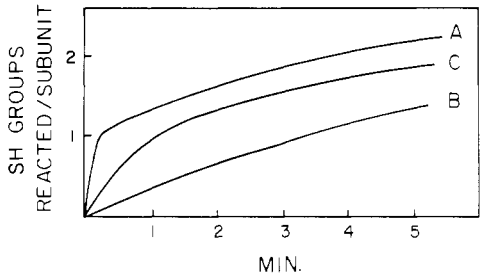

FIGURE 6: Reaction of thiols in phosphofructokinase with DTNB in the presence of ligands at pH 7.0. See text for conditions. (A) No additions; (B) plus 0.25 mM ATP; (C) plus 0.25 mM ATP and either 2.3 μM Fru-2,6-P_2, 40 μM Fru-1,6-P_2, or 220 μM Glu-1,6-P_2 (superimposible). Higher concentrations of bisphosphates had no further effect. The concentration of phosphofructokinase subunits was 2.3 μM. The concentration of DTNB used was 5 μM.

Effect of Sugar Bisphosphates on Reaction of Thiol Groups of Phosphofructokinase with DTNB. At pH 8.5, fructose-6-P and AMP decreased the rate of reaction of thiol groups of phosphofructokinase with DTNB, as previously described by Kemp & Forest (1968). Protection of thiol groups against reaction with DTNB was enhanced as the concentration of effector was increased, but the effects of fructose-6-P or AMP were clearly apparent at 0.1 mM effector. In contrast, fructose-1,6-P_2 had little or no effect on SH reactivity at concentrations up to 1 mM (data not shown). The same was true of fructose-2,6-P_2 at concentrations as high as 0.55 mM, the highest concentration tested. Kemp (1969) showed that at pH 7.0, MgATP protected the most reactive thiol group of the phosphofructokinase subunit against reaction with DTNB and that this protection was counteracted by phosphofructokinase activators (AMP, cyclic AMP, P_i, high pH). As shown in Figure 6, the phosphofructokinase activators fructose-2,6-P_2, fructose-1,6-P_2, and glucose-1,6-P_2 also counteracted this protection, probably by reducing the affinity of MgATP for the inhibitory site on the enzyme, as proposed (Kemp, 1969) for the other phosphofructokinase activators. The degree of counteraction of protection was the same at saturating concentrations of all three sugar bisphosphates (Figure 6).

Effect of Sugar Bisphosphates on Kinetics of Phosphorylation of Phosphofructokinase. It is known that the initial rate of phosphorylation of muscle (Kemp et al., 1981) and liver (Pilkis et al., 1982b) phosphofructokinase is increased by allosteric activators. In the present study, as shown in Table II, the sugar bisphosphates fructose-2,6-P_2, fructose-1,6-P_2, and glucose-1,6-P_2 all enhanced the rate of phosphofructokinase phosphorylation by the catalytic subunit of cyclic AMP

Table II: Initial Rate of Phosphorylation of Phosphofructokinase by the Catalytic Subunit of cAMP-Dependent Protein Kinase in the Presence of Various Ligands

ligand	% control rate
22 μM Fru-2,6-P_2	277
22 μM Fru-2,6-P_2 + 1 mM citrate	286
22 μM Fru-2,6-P_2 + 5 mM citrate	130
22 μM Fru-2,6-P_2 + 10 mM citrate	34
50 μM Fru-1,6-P_2	263
100 μM Fru-1,6-P_2	270
100 μM Fru-1,6-P_2 + 50 μM citrate	143
100 μM Fru-1,6-P_2 + 100 μM citrate	115
200 μM Fru-1,6-P_2 + 1 mM citrate	70
560 μM Glu-1,6-P_2	260
1.1 mM Glu-1,6-P_2 + 1 mM citrate	47

[a] See text for conditions. The concentration of phosphofructokinase subunits was approximately 22 μM. Higher concentrations of fructose 2,6-bisphosphate than that shown did not further increase the phosphorylation rate.

dependent protein kinase to the same maximum extent. It has been shown previously that of other activators of phosphofructokinase, AMP activates phosphorylation but to a lesser extent and P_i, another activator of phosphofructokinase, has no effect on phosphorylation of phosphofructokinase (Kemp et al., 1981). It was also shown previously that citrate inhibits phosphofructokinase phosphorylation (Kemp et al., 1981). As shown in Table II, 1 mM citrate inhibits phosphofructokinase phosphorylation even in the presence of 200 μM fructose-1,6-P_2 or 1.1 mM glucose-1,6-P_2. At lower citrate concentrations, the rate of phosphofructokinase phosphorylation in the presence of fructose-1,6-P_2 is greater than the control rate but is still less than that seen in the absence of citrate. In contrast, fructose-2,6-P_2 is far more potent in reversing the citrate effect. For example, 1 mM citrate had no effect on phosphofructokinase phosphorylation in the presence of a stoichiometric concentration (22 μM) of fructose-2,6-P_2. Even at 5 mM citrate, the rate of phosphorylation in the presence of 22 μM fructose-2,6-P_2 was still greater than the control rate.

Discussion

Quantitative measurement of activation of phosphofructokinase by fructose-1,6-P_2 is difficult because fructose-1,6-P_2 is either used up in the aldolase-coupled assay or accumulates in the pyruvate kinase–LDH-coupled assay. We have utilized two approaches for obviating this problem. First, we have used a pyruvate kinase–LDH-coupled assay with aldolase present to "buffer" the concentration of fructose-1,6-P_2 according to Tornheim & Lowenstein (1976). However, calculations indicate that during the course of a reaction where the concentration of substrate is reduced by only 5 μM (which is close to the practical limit of the spectrophotometric assay), the concentration of fructose-1,6-P_2 will still increase by about 1–3 μM (depending on the initial concentration of fructose-1,6-P_2), despite the presence of aldolase. This is enough to give some activation of the phosphofructokinase during the course of the assay, so the result will not be completely accurate. The second approach involves abandoning the enzyme-coupled spectrophotometric assay and using a highly sensitive direct radioactive assay to measure the rate of reaction before an activating concentration of product fructose-1,6-P_2 accumulates. We have found that reaction rates can be measured accurately under conditions where fructose-1,6-P_2 accumulates to an extent of 0.2–0.8 μM during the assay. Under these conditions, the amount of activation of phosphofructokinase by fructose-1,6-P_2 during the assay is minor. Of course, fructose-2,6-P_2 and glucose-1,6-P_2 can still be compared as phosphofructokinase activators by the aldolase-coupled spectrophotometric assay, since neither of these sugar bisphosphates are substrates for aldolase.

Therefore, we have used three different assay systems altogether: an aldolase-coupled spectrophotometric assay (assay A), a pyruvate kinase–LDH-coupled spectrophotometric assay (assay B), and a direct radioactive assay (assay C). Results from these three assay systems were consistent with one another, with fructose-2,6-P_2 having about 9-fold higher apparent affinity for phosphofructokinase than fructose-1,6-P_2 and about 50–100-fold higher affinity for phosphofructokinase than glucose-1,6-P_2. Equilibrium binding experiments also indicated that fructose-2,6-bisphosphate binds phosphofructokinase with about 1 order of magnitude greater affinity than fructose-1,6-P_2.

These results are in contrast to the 1000-fold higher binding affinity of fructose-2,6-P_2 compared to fructose-1,6-P_2 reported for rat liver phosphofructokinase (Van Schaftingen et al., 1981). The liver isozyme is apparently much less sensitive to activation by fructose-1,6-P_2 and glucose-1,6-P_2 than the muscle isozyme. This property could be related to possible differences in regulatory roles of various sugar bisphosphates for different phosphofructokinase isozymes found in different tissues. In rat liver, for example, the key role of fructose-2,6-P_2 in the regulation of phosphofructokinase activity is well established. In rat skeletal muscle, however (Hue et al., 1982), the concentration of fructose-2,6-P_2 (0.43 nmol/g) is sufficient to bind only 8% of the phosphofructokinase subunits (5 nmol/g) present (Tornheim & Lowenstein, 1976). In contrast, the concentration of fructose-1,6-P_2 in rat skeletal muscle was reported to be 65 nmol/g (Veech et al., 1969). These facts, together with the lack of correlation between fructose-2,6-P_2 levels and the glycolytic rate in perfused rat hind limb under various conditions (Hue et al., 1982), rule out a role for fructose-2,6-P_2 in regulation of phosphofructokinase activity in muscle but do not rule out a significant role for fructose-1,6-P_2.

Our equilibrium binding experiments strongly suggest that fructose-2,6-P_2 and fructose-1,6-P_2 bind to the same site on muscle phosphofructokinase. First, both compounds bound phosphofructokinase with negative cooperativity, unlike other phosphofructokinase ligands previously tested under the same conditions. Second, the binding of fructose-2,6-P_2 was inhibited in the presence of fructose-1,6-P or glucose-1,6-P_2 and vice versa. Third, both fructose-2,6-P_2 and fructose-1,6-P_2 inhibited the binding of MgATP and enhanced the binding of cyclic AMP to phosphofructokinase.

The idea that fructose-2,6-P_2 and fructose-1,6-P_2 indeed bind to a single site was further supported by a variety of experiments indicating that the conformation assumed by phosphofructokinase in the presence of fructose-2,6-P_2 is similar to that assumed in the presence of fructose-1,6-P_2. First sucrose gradient centrifugation indicated that the aggregation state of phosphofructokinase was the same in the presence of either fructose bisphosphate and different from that seen in the presence of another phosphofructokinase activator, AMP. Second, neither fructose bisphosphate protected any thiol groups of phosphofructokinase against reaction with DTNB at pH 8.5, unlike fructose-6-P and AMP, but both fructose bisphosphates counteracted the effect of MgATP on thiol reactivity at pH 7.0. Third, both fructose bisphosphates activated the phosphorylation of phosphofructokinase in vitro to the same maximal extent, unlike AMP and P_i, although the enzyme appeared to have lower affinity for the inhibitor citrate when bound by fructose-2,6-P_2 than when bound by fruc-

tose-1,6-P_2. This latter observation suggests very subtle differences in the conformations of phosphofructokinase when bound by fructose-2,6-P_2 and when bound by fructose-1,6-P_2. Binding of fructose-2,6-P_2 may induce a "less inhibitable" subconformation due to a better fit at the sugar bisphosphate binding site.

In those conformational experiments where glucose-1,6-P_2 was also tested (i.e., thiol reactivity at pH 7.0 and in vitro phosphorylation activation), its action proved to be similar to that of the fructose bisphosphates. These results are consistent with the binding data, which indicate that glucose-1,6-P_2 binds to the common sugar bisphosphate site on muscle phosphofructokinase.

Phosphofructokinase from Ehrlich ascites tumor cells (Bosca et al., 1982) appears to be completely insensitive to activation by fructose-1,6-P_2 but is activated by fructose-2,6-P_2. This led Bosca et al. to speculate that fructose-2,6-P_2 and fructose-1,6-P_2 have distinct binding sites on phosphofructokinase and that the Ehrlich ascites tumor cell phosphofructokinase lacks the fructose-1,6-P_2 site. However, it is not necessary, in this instance, to invoke separate sites of action for the two fructose bisphosphates. We think it more likely that the ascites tumor isozyme possesses a single sugar bisphosphate binding site, which happens to have significant affinity for fructose-2,6-P_2 only. The tumor isozyme may be similar in this regard to the human F_4 isozyme that was reported to be completely insensitive to activation by glucose-1,6-P_2 (Meienhofer et al., 1980). Because of the existence of these fructose-1,6-P_2- and glucose-1,6-P_2-insensitive isozymes, we propose that, in general, the "sugar bisphosphate" binding site be thought of primarily as a fructose-2,6-P_2 binding site that has a greater or lesser degree of specificity, depending upon isozymic or (possibility) species differences.

In summary, we conclude that fructose-2,6-P_2, fructose-1,6-P_2, and glucose-1,6-P_2 bind to a common site of action on muscle phosphofructokinase, referred to as the fructose-2,6-P_2 site. Assuming that the same conclusion holds for other phosphofructokinase isozymes, it appears that the fructose-2,6-P_2 site of muscle phosphofructokinase is less specific for fructose-2,6-P_2 than the corresponding site in other phosphofructokinase isozymes, a finding consistent with the probable lack of importance of fructose-2,6-P_2 in regulation of phosphofructokinase activity in skeletal muscle.

Registry No. Fructose-2,6-P_2, 77164-51-3; fructose-1,6-P_2, 488-69-7; glucose-1,6-P_2, 10139-18-1; fructose-2,6-[2-^{32}P]P_2, 86595-18-8; fructose-1,6-[1-^{32}P]P_2, 71595-29-4; glucose-1,6-[6-^{32}P]P_2, 86610-13-1; phosphofructokinase, 9001-80-3.

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