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An Equilibrium Binding Study of the Interaction of Fructose 6-Phosphate and Fructose 1,6-Bisphosphate with Rabbit Muscle Phosphofructokinase†

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ABSTRACT: Equilibrium binding studies of the interaction of rabbit muscle phosphofructokinase with fructose 6-phosphate and fructose 1,6-bisphosphate have been carried out at 5° in the presence of 1-10 mM potassium phosphate (pH 7.0 and 8.0), 5 mM citrate (pH 7.0), or 0.22 mM adenylyl imidodiphosphate (pH 7.0 and 8.0). The binding isotherms for both fructose 6-phosphate and fructose 1,6-bisphosphate exhibit negative cooperativity at pH 7.0 and 8.0 in the presence of 1-10 mM potassium phosphate at protein concentrations where the enzyme exists as a mixture of dimers and tetramers (pH 7.0) or as tetramers (pH 8.0) and at pH 7.0 in the presence of 5 mM citrate where the enzyme exists primarily as dimers. The enzyme binds 1 mol of either fructose phosphate/mol of enzyme monomer (molecular weight 80,000). When enzyme aggregation states smaller than the tetramer are present, the saturation of the enzyme with either ligand is paralleled by polymerization of the enzyme to tetramer, by an increase in enzymatic activity and by a quenching of the protein fluorescence. At protein concentrations where aggregates higher than the tetramer predominate, the fructose 1,6-bisphosphate binding isotherms are hyperbolic. These results can be quantitatively analyzed in terms of a model in which the dimer is associated with extreme negative cooperativity in binding the ligands, the tetramer is associated with less negative cooperativity, and aggregates larger than the tetramer are associated with little or no cooperativity in the binding process. Phosphate is a competitive inhibitor of the fructose phosphate sites at both pH 7.0 and 8.0, while citrate inhibits binding in a complex, noncompetitive manner. In the presence of the ATP analog adenylyl imidodiphosphate, the enzyme-fructose 6-phosphate binding isotherm is sigmoidal at pH 7.0, but hyperbolic at pH 8.0. The characteristic sigmoidal initial velocity-fructose 6-phosphate isotherms for phosphofructokinase at pH 7.0, therefore, are due to an heterotropic interaction between ATP and fructose 6-phosphate binding sites which alters the homotropic interactions between fructose 6-phosphate binding sites. Thus the homotropic interactions between fructose 6-phosphate binding sites can give rise to positive, negative, or no cooperativity depending upon the pH, the aggregation state of the protein, and the metabolic effectors present. The available data suggest the regulation of phosphofructokinase involves a complex interplay between protein polymerization and homotropic and heterotropic interactions between ligand binding sites.

Rabbit skeletal muscle phosphofructokinase is activated and inhibited by a number of metabolites (Passoneau and Lowry, 1962, 1963). The enzyme exhibits concentration- and pH-dependent aggregation (Pavelich and Hammes, 1973; Aaronson and Frieden, 1972; Leonard and Walker, 1972) as well as sigmoidal initial velocity-fructose 6-phosphate concentration isotherms (Hofer and Pette, 1968). Moreover, a correlation exists between the enzymes' aggregation state and its specific activity in the presence of various activators and inhibitors (Lad *et al.*, 1973). At an enzyme concentration of 0.15 mg/ml and pH 7.0, strong activators such as fructose 1,6-bisphosphate stabilize fully ac-

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tive tetramers, whereas citrate, a potent inhibitor of the enzyme, stabilizes inactive dimers (Lad *et al.*, 1973). At protein concentrations of approximately 1 mg/ml, pH 7.0, the enzyme exists as aggregates larger than the tetramer (Pavelich and Hammes, 1973), and the binding of fructose 6-phosphate to the enzyme is characterized by a hyperbolic binding isotherm (Kemp and Krebs, 1967). The sigmoidal initial velocity–fructose 6-phosphate isotherm could arise in several different ways: (1) an heterotropic interaction between MgATP and fructose 6-phosphate binding sites; (2) protein aggregation where the enzyme is shifted from an inactive form (dimer) to an active form (tetramer) by fructose 6-phosphate; and (3) a kinetic phenomenon where special relationships among the rate constants lead to a complex initial velocity–fructose 6-phosphate isotherm.

In this study the binding of fructose 6-phosphate and fructose 1,6-bisphosphate to rabbit muscle phosphofructokinase was investigated under conditions where the enzyme exists mainly as dimer, tetramer, or higher aggregates. The binding isotherms for the binding of fructose 6-phosphate and fructose 1,6-bisphosphate to the enzyme exhibit negative cooperativity in the presence of potassium phosphate at a protein concentration where the enzyme exists as dimer and tetramer (pH 7.0) or as tetramer (pH 8.0) and at pH 7.0 in the presence of citrate where the enzyme exists primarily as dimer. The saturation of mixtures of dimeric and tetrameric enzyme with either ligand is paralleled by conversion of the enzyme dimers to tetramers. At a protein concentration where aggregates higher than tetramer predominate (0.75 mg/ml, pH 8.0), the fructose 1,6-bisphosphate binding isotherm is hyperbolic. These results can be quantitatively explained by a model in which the binding of ligands to the dimer is associated with extreme negative cooperativity, binding to the tetramer is associated with less negative cooperativity, and binding to aggregates larger than the tetramer displays little or no cooperativity. At pH 7.0, in the presence of the ATP analog adenylyl imidodiphosphate (AMP-PNP;[1] Yount *et al.*, 1971), the fructose 6-phosphate binding isotherm is sigmoidal. Thus the sigmoidal kinetic isotherms apparently are the result of a heterotropic interaction between ATP and fructose 6-phosphate binding sites, which alters the homotropic interaction between fructose 6-phosphate binding sites. These homotropic interactions can give rise to positive, negative, or no cooperativity, depending on the experimental conditions.

Experimental Section

Materials. The ATP, fructose 6-phosphate, fructose 1,6-bisphosphate, Tris base, imidazole, ethylenediaminetetraacetic acid, dithiothreitol, α-glycerol phosphate, aldolase, α-glycerolphosphate dehydrogenase, triosephosphate isomerase, phosphoglucoisomerase, glucose-6-phosphate dehydrogenase, and bovine serum albumin were obtained from Sigma Chemical Co. Imidazole was recrystallized twice from benzene. Fructose 6-phosphate and fructose 1,6-bisphosphate were purified on Dowex 1-borate ion exchange columns (1 cm i.d. × 25 cm) (Williams *et al.*, 1971) or a Dowex 1-Cl ion exchange column (1 cm i.d. × 25 cm) (Benson, 1957). Radioactive [^{14}C]fructose 6-phosphate (213 Ci/mol) and [^{14}C]fructose 1,6-bisphosphate (250 Ci/mol) were obtained from Amersham/Searle and were purified by paper chromatography in 95% ethanol–0.8% acetic acid (1:1) (pH 3.5) and elution of the appropriate parts of the chromatogram with water. The eluted radioactive fructose phosphate was lyophilized, dissolved in a small volume of water, and frozen for storage. Glycylglycine was obtained from Calbiochem. Adenylyl imidodiphosphate (AMP-PNP) and β,γ-methyleneadenosine 5′-triphosphate were purchased from P-L Biochemicals and used without further purifications. The scintillation fluors were obtained from Packard and dioxane was 99 mol %. Diaflow XM-50 membranes were obtained from Amnicon Corporation. All other chemicals and reagents were the best available commercial products. Distilled deionized water was used in all experiments.

Phosphofructokinase. Rabbit skeletal muscle phosphofructokinase was purified by the method of Ling *et al.* (1966). The final ammonium sulfate precipitate was dissolved in pH 8.0, 0.1 M potassium phosphate and 1 mM ethylenediaminetetraacetic acid, and dialyzed against the same buffer to give a stock solution of 10–15 mg/ml. The enzyme concentration was determined from the absorbance at 280 nm using an extinction coefficient of 1.02 ml/(mg cm) (Parmeggiani *et al.*, 1966). The specific activity of the enzyme at 23° in 0.1 M potassium phosphate (pH 8.0) was 110–120 units/mg, where a unit of enzyme activity is defined as the production of 1 μmol of product/min. After a period of about 4 weeks, the specific activity of the enzyme stock solution declines significantly, and the stock solution becomes opaque. Only enzyme with a specific activity greater than 100 units/mg was employed in this study. In equilibrium binding experiments, where it was necessary to achieve low phosphate concentrations at relatively high protein concentrations, stock solutions of enzyme were dialyzed into an appropriate lower concentration of potassium phosphate (never lower than 25 mM), pH 8.0, the day prior to use. The results reported here were obtained with six different enzyme preparations.

Steady-State Kinetics. The production of fructose 1,6-bisphosphate by the phosphofructokinase reaction was followed with the coupled enzyme reactions of aldolase, triosephosphate isomerase, and α-glycerolphosphate dehydrogenase by measuring the disappearance of NADH spectrophotometrically (Ling *et al.*, 1966). The assays were carried out under the following conditions: pH 8.0, 33 mM Tris-Cl, 2 mM ATP, 5 mM $MgCl_2$, 2 mM fructose 6-phosphate, 0.1 mM NADH, 1 mM dithiothreitol, 0.25 unit/ml of aldolase, 3.2 units/ml of α-glycerolphosphate dehydrogenase, 35 units/ml of triosephosphate isomerase, and 0.1–0.3 μg of phosphofructokinase in a total volume of 3 ml. The assays were initiated by the addition of phosphofructokinase to the assay mixture, and the velocity of the enzymatic reaction was recorded spectrophotometrically at 340 nm using a Cary 14 spectrophotometer thermostated at 23°. Assay velocities were unaltered by increasing the auxiliary enzyme concentrations, and the NADH concentration was sufficiently low to avoid inhibition of the auxiliary enzymes (Newsholme *et al.*, 1970).

The inhibition of the steady-state initial velocity by AMP-PNP and β,γ-methyleneadenosine 5′-triphosphate was determined over an ATP concentration range of 6–50 μM and inhibitor concentration ranges of 22–122 μM.

Gel Chromatography. Column chromatography on agarose (1.5-m resin, Bio-Rad Laboratories; 1.5 cm i.d. × 50 cm) was used to determine the Stokes' radius of phosphofructokinase. The top and bottom of each column contained 0.8-cm plugs of Sephadex G-25 to ensure stable agarose in-

[1] Abbreviations used are: AMP-PNP, adenylyl imidodiphosphate; Fru-1,6-P_2, fructose 1,6-bisphosphate; Fru-6-P, fructose 6-phosphate.

terfaces. The concentration of enzyme was 0.15 or 0.75 mg/ml, and all elutions were carried out at 5°. Elution profiles were analyzed by measuring the absorbance of the eluate at 280 nm. The details of the experimental procedure and the analysis of the results were as previously described (Pavelich and Hammes, 1973; Lad *et al.*, 1973).

Fluorescence Measurements. Fluorescence titrations of phosphofructokinase with ligands and fluorescence polarization measurements were performed with a Hitachi Perkin-Elmer MPF-3 fluorescence spectrophotometer thermostated at 5°. The protein was excited at 280 nm and changes in the emission at 328 nm were monitored. The excitation and emission slits were 4 nm. Since the total change in the relative fluorescence was only 5–9%, the titrations were performed using a fivefold scale expansion and a linear offset potential. Changes in fluorescence were corrected for dilution by the added ligand. The concentration of the stock ligand solution was adjusted so that these corrections were never larger than 2% of the relative fluorescence.

Fructose 6-Phosphate and Fructose 1,6-Bisphosphate Determinations. The concentrations of labeled and unlabeled fructose 6-phosphate solutions were determined with the coupled reactions of phosphoglucoisomerase and glucose-6-phosphate dehydrogenase (Hohorst, 1963). The concentrations of labeled and unlabeled fructose 1,6-bisphosphate solutions were determined by the coupled reactions of aldolase, triosephosphate isomerase, and α-glycerolphosphate dehydrogenase (Bücher and Hohorst, 1963). The specific radioactivity of each ligand solution was then obtained by determination of the radioactivity of a known concentration of the ligand. Radioactivity determinations were carried out in Bray's scintillation fluid (Bray, 1960) and counted with a Beckman LS-255 liquid scintillation counter. Radioactive samples were counted to a relative precision of 1% or greater at the 95% confidence level. Counting rates were converted to disintegration rates by using standard quenching curves for the scintillation fluid and the scintillation counter discriminators.

Equilibrium Binding Measurements. Binding measurements were done at 5° using the "forced dialysis" technique (Cantley and Hammes, 1973). Solutions of varying radioactive ligand concentration, effector, protein, and buffer were mixed in 0.5-ml portions and incubated 2 hr at 5°. Longer incubation had no effect upon the amount of ligand bound to the protein, indicating equilibrium had been achieved. The total ligand concentration of each solution was determined by measuring the radioactivity of a 10-μl sample and utilizing the known ligand specific radioactivity. The solutions were then placed in a Metalloglass ultrafiltration cell and a pressure of 25–40 psi of nitrogen was applied for 10–15 min until 10–15 μl of solution was forced through the Diaflow XM-50 membranes; 10-μl aliquots of the free ligand solution were collected from the underside of the membranes and the radioactivity was determined. The bound ligand concentration was calculated as the difference between the total and free ligand concentrations. Repeated measurements have indicated that this technique has a relative error in the determination of either the total or free ligand concentration of approximately 3%. Control experiments showed that the ligands used in this study were not bound by the XM-50 membranes and that phosphofructokinase does not pass through the membranes. Since the protein was concentrated only 2–4% during the experiment, strict control of the enzyme aggregation state was possible.

Analysis of Binding Data. Binding data were fit to various mechanistic nonlinear equations using a weighted nonlinear least-squares procedure devised by Marquardt (1963). Binding data were fit in the functional form of $r/(S)$ (dependent variable) *vs.* (S) (independent variable) where r is the ratio of the concentration of bound ligand to the enzyme concentration and (S) is the free ligand concentration. Individual data points were weighted unequally as

$$w_i = [\sigma r_i + \sigma(S_i)]/(S_i)^2$$

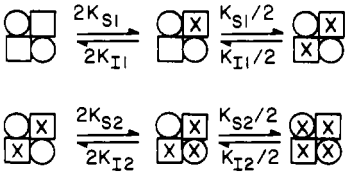
where the w_i are the weighting factors for the ith data point and σ is the relative error in r or (S) (Beers, 1957). The value of σ was assumed to be 0.03, which is the relative standard error of each determination. At the minimum, the weighted sum of squares was tested as a chi-square variable, χ^2, with ν degrees of freedom (Bevington, 1969). A fit was judged acceptable if $\chi^2/\nu \leq 1$, and all of the data fits presented in the Results satisfied this criterion. Although this test is not exact with nonlinear model equations, it is widely used nevertheless (Bevington, 1969). The relative errors of fitted parameters reported in this paper were computed using the linear approximation (Chandler *et al.*, 1972) and can only be taken as approximate estimates since the equations are nonlinear.

Results

Fructose Phosphate Binding at pH 8. The binding isotherms for fructose 1,6-bisphosphate and fructose 6-phosphate binding to phosphofructokinase at a concentration of 0.15 mg/ml at various phosphate concentrations, pH 8.0, are shown as plots of $r/(S)$ *vs.* r in Figure 1A and B, respectively (Scatchard, 1949). The value of r, the ratio of the concentration of bound ligand to the enzyme concentration, was calculated in terms of the concentration of enzyme monomer, assuming a monomer molecular weight of 80,000 g/mol (Leonard and Walker, 1972; Aaronson and Frieden, 1972; Pavelich and Hammes, 1973); (S) is the free ligand concentration. Under these conditions the enzyme is largely tetrameric (Pavelich and Hammes, 1973). These plots indicate that the binding cannot be characterized by a single binding constant. Instead the binding apparently becomes weaker as the fraction of enzyme sites occupied increases. This type of binding isotherm can be due to interactions between equivalent binding sites ("negative cooperativity"; Koshland, 1969) or to the presence of two or more different classes of sites with different intrinsic association constants.

Potassium phosphate dramatically affects the binding isotherms by competing for the fructose phosphate sites. Initial attempts to perform the binding experiments in 0.1 M potassium phosphate showed the occurrence of a large amount of relatively weak, apparently nonspecific, binding. Values of r as high as 57 were observed. Increasing the protein concentration reduced the nonspecific binding somewhat, but did not eliminate it. Attempts to perform the binding experiments in the presence of 25 mM α-glycerol phosphate (Kemp and Krebs, 1967) at 0.15 mg of phosphofructokinase/ml resulted in very weak binding, and α-glycerol phosphate also was found to compete for the specific fructose phosphate binding sites (unpublished results).

The data of Figure 1A and B can be quantitatively analyzed according to the model illustrated in I. This model postulates that a dimer of enzyme has two different classes of binding sites for phosphate and fructose phosphates, each class of sites characterized by an intrinsic binding constant for each ligand, and that binding to the tighter class of binding sites occurs before binding to the weaker class of



I

sites can occur. (Negative cooperativity due to interactions between sites is an equivalent model.) This model is not unique, but has the virtue of involving a relatively small number of parameters and of being relatively simple conceptually, while at the same time providing a good description of the data. The two requisites of any model are an apparent decrease in binding affinity as the ligand concentration increases and the competition of phosphate and the fructose phosphates for the same sites on the enzyme. In I the symbol X represents either the fructose phosphates binding to the enzyme with association constants K_{S1} and K_{S2} or phosphate binding with association constants K_{I1} and K_{I2}. That is, phosphate is a competitive inhibitor of fructose phosphase binding. For the model in I, r is given by eq A1 (Appendix). The maximum number of moles of ligand bound per mole of protein (molecular weight 80,000) was assumed to be 1, which is the approximate value to which all of the plots in Figure 1 extrapolate. The data were fit to eq A1 by a least-squares computer analysis and the parameters obtained are compiled in Table I; the solid lines in Figure 1 are the curves calculated from eq A1 and the parameters in Table I. The data at two potassium phosphate concentrations were fit simultaneously. The average relative deviation in the parameters ranged from 1 to 20%. The ratio of K_{S1} for fructose 1,6-bisphosphate to K_{S1} for fructose 6-phosphate is 3.5 while the ratio of K_{S2} for fructose 1,6-bisphosphate to K_{S2} for fructose 6-phosphate is 4.3. Thus fructose 1,6-bisphosphate binds about four times more tightly than fructose 6-phosphate to the enzyme under these conditions. The ratio of K_{I1} from the fructose 1,6-bisphosphate binding data to K_{I1} from the fructose 6-phosphate binding data is 1.2 while the same ratio for the values of K_{I2} from the two sets of data is 0.88. These ratios are sufficiently close to unity to indicate the data are consistent with and are well described by the model used.

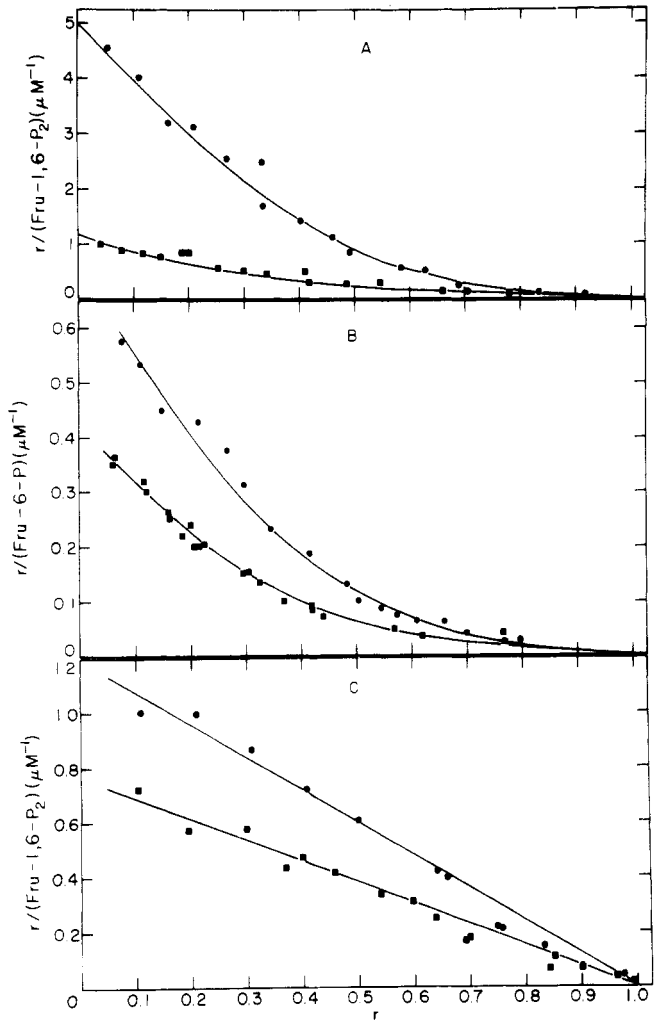


FIGURE 1: Plots of $r/(S)$ *vs.* r, where r is the moles of ligand bound per mole of enzyme (80,000 molecular weight) and (S) is the free ligand concentration, for the binding of fructose 1,6-bisphosphate (Fru-1,6-P_2) (A and C) and fructose 6-phosphate (Fru-6-P) (B) at 5° and pH 8.0 (25 mM glycylglycine, 5 mM dithiothreitol, and 1 mM ethylenediaminetetraacetic acid) to 0.15 mg of phosphofructokinase/ml (A and B) and to 0.75 mg of phosphofructokinase/ml (C). Potassium phosphate concentrations were (●) 2 mM and (■) 10 mM for fructose 1,6-bisphosphate binding in A, (●) 3.6 mM and (■) 6.6 mM for fructose 6-phosphate binding in B, and (●) 8 mM and (■) 13 mM for fructose 1,6-bisphosphate binding in C. The solid curves were calculated from eq A1 and the parameters in Table I for A and B, and from eq 1 and the parameters in Table I for C. For each ligand the parameters of Table I were obtained by a simultaneous least-squares fitting of the data for the two potassium phosphate concentrations. The total ligand concentration varied from 0.1 to 90 μM for fructose 1,6-bisphosphate and 0.2 to 50 μM for fructose 6-phosphate.

Table I: Parameters for Fructose 1,6-Bisphosphate and Fructose 6-Phosphate Binding to Phosphofructokinase in Potassium Phosphate, pH 8.0.

Ligand	Enzyme (mg/ml)	K_{S1} (μM^{-1})	K_{S2} (μM^{-1})	K_{I1} (mM^{-1})	K_{I2} (mM^{-1})
Fru-1,6-P_2	0.15[a]	27.3	0.502	1.18	0.372
Fru-6-P	0.15[a]	7.87	0.118	1.42	0.424
Fru-1,6-P_2	0.75[b]	7.88		0.696	

[a] The data in Figure 1A and B were fit to eq A1. The experiments were carried out in 25 mM glycylglycine (pH 8.0), 5 mM dithiothreitol, and 1 mM ethylenediaminetetraacetic acid, 5°. [b] The data in Figure 1C were fit to eq 1. The experiments were carried out in 25 mM glycylglycine (pH 8.0), 5 mM dithiothreitol, and 1 mM ethylenediaminetetraacetic acid, 5°.

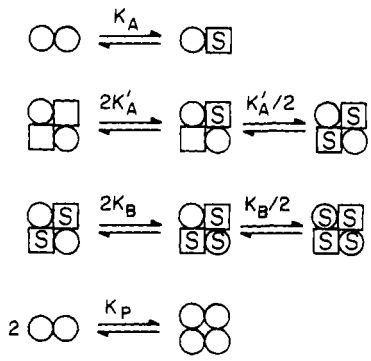
Figure 1C shows the effect of increasing the protein concentration to 0.75 mg/ml (from 0.15 mg/ml) on the binding of fructose 1,6-bisphosphate to the enzyme at pH 8.0. Under these conditions, the enzyme exists predominantly as aggregates larger than the tetramer (Pavelich and Hammes, 1973; Aaronson and Frieden, 1972). The protein now appears to bind the ligand as n sets of identical independent sites with potassium phosphate still competing for the fructose 1,6-bisphosphate binding sites. The data in Figure 1C were fit to eq 1 which assumes equivalent, independent binding sites, competitive inhibition between phosphate and fructose 1,6-bisphosphate, and a maximum enzyme capacity of 1 mol of ligand/mol of monomer (molecular weight of 80,000). The results obtained from the nonlinear least-squares analysis are presented in Table I. The lines in Figure 1C have been computed with eq 1 and the

$$r = \frac{K_{S1}(S)}{1 + K_{S1}(S) + K_{I1}(I)} \qquad (1)$$

parameters in Table I. The average relative parameter deviation was 1% for K_{S1} and 24% for K_{I1}.

Fructose Phosphate Binding at pH 7 in Potassium Phosphate. Figure 2A shows the binding of fructose 1,6-bisphosphate to 0.15 mg of phosphofructokinase/ml at various potassium phosphate concentrations and Figure 2B shows the binding of fructose 1,6-bisphosphate to various concentrations of phosphofructokinase at 2 mM potassium phosphate. The binding curves are similar to those at pH 8.0. However, in incubation mixtures of 0.15 mg of phosphofructokinase/ml, 2 mM potassium phosphate, 25 mM imidazole-Cl (pH 7.0), 1 mM ethylenediaminetetraacetic acid, and 5 mM dithiothreitol, the enzyme has a Stokes' radius of 55 Å and a specific activity of 60.2 units/mg. This indicates a considerable amount of dimer is present (Lad *et al.*, 1973). In the presence of 1 mM fructose 1,6-bisphosphate the enzyme specific activity increases to 128 units/mg, indicating polymerization to the tetramer when the enzyme is saturated with ligand (Lad *et al.*, 1973).

The binding of fructose 6-phosphate to the enzyme under these conditions was again similar to fructose 1,6-bisphosphate binding (Figure 2C) but the degree of negative cooperativity appears to be less. In order for any mechanism to adequately fit the above data, it must allow for enzyme polymerization and a negatively cooperative binding isotherm. One such mechanism is shown in II. The essential features



II

of this mechanism are the following. (1) Binding to the dimer is associated with extreme negative cooperativity so that over the range of ligand concentrations used, only one ligand molecule is bound to the dimer with association constant K_A.(2) The tetramer may bind four ligand molecules with two intrinsic association constants K_A' and K_B, *i.e.*, negative cooperativity occurs in binding to the dimer unit, and binding to the tight sites occurs prior to binding to the weak sites. (3) The dimer may polymerize to tetramer with association constant K_P. All other polymerizations may be written in terms of the four constants, K_A, K_A', K_B, and K_P. The model (II) has been cast in terms of two types of preexisting sites; however, the assumption of equivalent sites with negative cooperativity leads to identical predictions. Further support for this mechanism is given by the data for fructose phosphate binding to the enzyme in the presence of 5 mM citrate (see below).

Each set of data in Figure 2 was individually fit to the model in II (eq A2) and the best fit parameters are presented in Table II. The average relative parameter deviation ranged from 1 to 57%. Increasing the potassium phosphate concentration lowers the ligand association constants as expected. The binding constants and the ratio K_A'/K_B appear to decrease somewhat as the protein concentration is increased. This may be due to the experimental errors, but

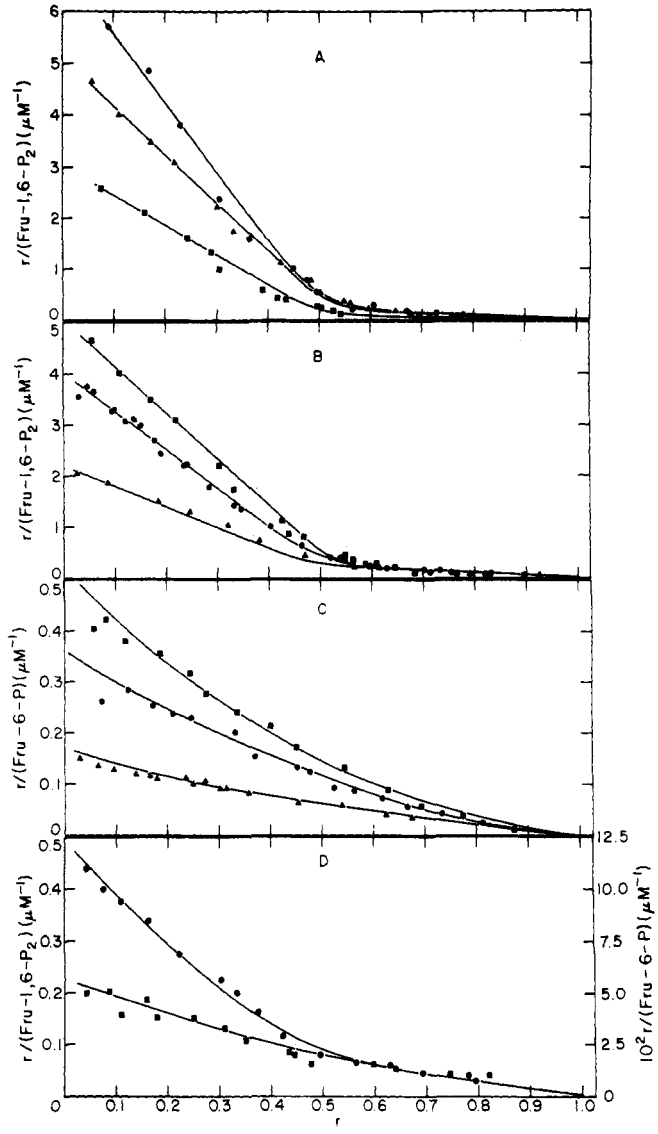


FIGURE 2: Plots of $r/(S)$ *vs.* r, where r is the moles of ligand bound per mole of enzyme (80,000 molecular weight) and (S) is the free ligand concentration for the binding of fructose 1,6-bisphosphate (Fru-1,6-P_2) (A, B, and D) and fructose 6-phosphate (Fru-6-P) (C and D) at 5° and pH 7.0 [25 mM imidazole-Cl (A-C) or 0.1 M Tris-Cl (D), 5 mM dithiothreitol, and 1 mM ethylenediaminetetraacetic acid] to phosphofructokinase. In A, the enzyme concentration was 0.15 mg/ml and potassium phosphate concentrations were (●) 1 mM, (▲) 2 mM, and (■) 6.4 mM. In B, the potassium phosphate concentration was 2 mM and the enzyme concentration was (■) 0.15 mg/ml, (●) 0.3 mg/ml, and (▲) 0.75 mg/ml. In C, the enzyme and potassium phosphate concentrations were respectively (■) 0.15 mg/ml and 2 mM, (●) 0.15 mg/ml and 3.6 mM, and (▲) 0.3 mg/ml and 3.6 mM. In D, the enzyme concentration was 0.15 (●) and 0.75 (■) mg/ml for fructose 1,6-bisphosphate and fructose 6-phosphate, respectively, in the presence of 5 mM citrate. The solid curves were calculated from eq A2 and the parameters in Tables II and III. The total ligand concentration varied between 0.2 and 50 μM for fructose 1,6-bisphosphate and between 0.2 and 90 μM for fructose 6-phosphate.

more likely is due to the presence of aggregates larger than the tetramer at high protein concentrations. At the same phosphate concentration, fructose 6-phosphate has smaller association constants than fructose 1,6-bisphosphate and the degree of negative cooperativity is less, *i.e.*, K_A'/K_B is smaller.

Fructose Phosphate Binding at pH 7 in the Presence of Citrate. The binding isotherm for fructose 1,6-bisphosphate (Fru-1,6-P_2) binding to 0.15 mg of phosphofructokinase/ml

Table II: Parameters for Fructose 1,6-Bisphosphate and Fructose 6-Phosphate Binding to Phosphofructokinase in Potassium Phosphate, pH 7.0.[a]

Ligand	Enzyme (mg/ml)	Phosphate (mM)	K_A (μM^{-1})	K_A' (μM^{-1})	K_B (μM^{-1})	K_P (μM^{-1})
Fru-1,6-P_2	0.15	1.0	13.2	27.5	0.695	0.015
Fru-1,6-P_2	0.15	2.0	10.1	24.5	0.454	0.015
Fru-1,6-P_2	0.15	6.4	5.88	12.7	0.266	0.014
Fru-1,6-P_2	0.3	2.0	7.70	21.4	0.591	0.0083
Fru-1,6-P_2	0.75	2.0	4.13	7.96	0.282	0.011
Fru-6-P	0.15	2.0	1.10	1.35	0.421	0.028
Fru-6-P	0.15	3.6	0.871	1.08	0.378	0.026
Fru-6-P	0.3	3.6	0.358	0.281	0.279	0.048

[a] The data in Figure 2 were fit to eq A2. All experiments were carried out in 25 mM imidazole-Cl (pH 7.0), 5 mM dithiothreitol, and 1 mM ethylenediaminetetraacetic acid, 5°.

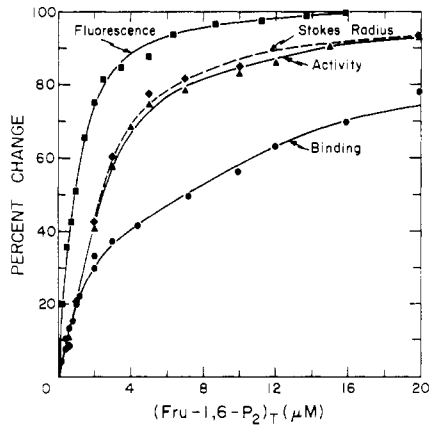


FIGURE 3: The per cent change in the properties of phosphofructokinase (incubated at 0.15 mg of phosphofructokinase/ml in 0.1 M Tris-Cl, pH 7.0, 5°, 5 mM citrate, 5 mM dithiothreitol, and 1 mM ethylenediaminetetraacetic acid) plotted *vs.* the total concentration of fructose 1,6-bisphosphate (Fru-1,6-P_2)$_T$. The per cent change in binding (●) is expressed as 100r, where r is the number of moles of ligand bound per mole of enzyme (molecular weight 80,000). Changes in specific activity (▲) are calculated as $100(A_x - A_0)/(A_\infty - A_0)$ where A_0 is the initial specific activity (20.8 units/mg), A_x is the specific activity at any given fructose 1,6-bisphosphate concentration, and A_∞ is the specific activity at 1 mM fructose 1,6-bisphosphate (130 units/mg). Assays were done at pH 8.0 (23°) using the standard assay described in the Experimental Section. The changes in Stokes' radius of the incubation mixtures (♦) were calculated as $100(R_s^x - R_s^0)/(R_s^\infty - R_s^0)$ where R_s^0 is the initial Stokes' radius (43.5 Å), R_s^x is the Stokes' radius at any given fructose 1,6-bisphosphate concentration, and R_s^∞ is the Stokes' radius of the tetramer (68 Å). Changes in protein fluorescence (■) are calculated as $100(F_0 - F_x)/(F_0 - F_\infty)$ where F_0 is the initial relative protein fluorescence, F_x is the relative protein fluorescence at a given fructose 1,6-bisphosphate concentration, and F_∞ is the relative protein fluorescence at 1 mM fructose 1,6-bisphosphate. The total change in the relative protein fluorescence was 9.6% at the 328-nm emission when the protein was excited at 280 nm. The solid curves were calculated from eq A2-A5 and the parameters in Table III.

in the presence of 5 mM citrate (pH 7.0) is shown in Figure 2D as a plot of r/(Fru-1,6-P_2) *vs.* r. The binding isotherm is similar to that of Figure 1A obtained at low potassium phosphate concentrations, pH 8.0. At pH 7.0 with low fructose 1,6-bisphosphate and high citrate concentrations, phosphofructokinase exists primarily as a dimer (Lad *et al.*, 1973). As the enzyme is saturated with fructose 1,6-bis-

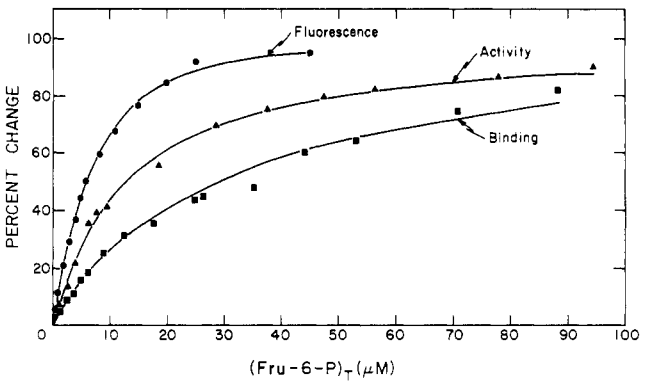


FIGURE 4: The per cent change in the properties of phosphofructokinase (incubated at 0.75 mg of phosphofructokinase/ml in 0.1 M Tris-Cl (pH 7), 5°, 5 mM citrate, 5 mM dithiothreitol, and 1 mM ethylenediaminetetraacetic acid) plotted *vs.* the total fructose 6-phosphate concentration (Fru-6-P)$_T$. The per cent change in binding (■) is expressed as 100r, where r is the number of moles of ligand bound per mole of enzyme (molecular weight 80,000). The changes in specific activity (▲) are calculated as $100(A_x - A_0)/(A_\infty - A_0)$ where A_0 is the initial specific activity (40.2 units/mg), A_x is the specific activity at any given fructose 6-phosphate concentration and A_∞ is the specific activity at 1 mM fructose 6-phosphate (130 units/mg). Assays were done at pH 8 (23°) using the standard assay described in the Experimental Section. Changes in protein fluorescence (●) are expressed as $100(F_0 - F_x)/(F_0 - F_\infty)$ where F_0 is the initial relative protein fluorescence, F_x is the relative protein fluorescence at a given fructose 6-phosphate concentration, and F_∞ is the relative fluorescence at 1 mM fructose 6-phosphate. The total change in the relative protein fluorescence was 5.8% at the 328-nm emission when the protein was excited at 280 nm. The Stokes' radius of the incubation mixture (0.75 mg of phosphofructokinase/ml) with no fructose 6-phosphate was 49 Å. The solid curves were calculated with eq A2, A3, and A6 and the parameters in Table III.

phosphate, the dimer polymerizes to a tetramer. Figure 3 shows the change in the Stokes' radius of the enzyme as determined by gel filtration with increasing fructose 1,6-bisphosphate concentration. The change in the specific activity of the enzyme as it aggregates also is shown in Figure 3. As previously shown, the changes in the enzymes' aggregation state correlate well with its specific activity (Lad *et al.*, 1973). For these activity measurements, the enzyme was incubated 2 hr at 5°, 0.15 mg/ml in 0.1 M Tris-Cl (pH 7.0), with 5 mM citrate and varying fructose 1,6-bisphosphate concentrations. The specific activity was then measured by dilution of the enzyme to 0.1 μg/ml in the standard assay mixture (see Experimental Section) at pH 8.0. The implicit assumption is made that activities measured in this manner reflect the aggregation state of the enzyme in the incubation mixture (Lad *et al.*, 1973). The assumption is supported by the rates for depolymerization of the tetramer (half-life of 1.5 hr) and dimer polymerization (half-life of less than 5 min at 0.15 mg of phosphofructokinase/ml) (Lad *et al.*, 1973). Dimer polymerization, which is dependent on protein concentration, would be very slow under the steady-state assay conditions (approximately 0.1 μg/ml of enzyme).

Phosphofructokinase protein fluorescence is quenched about 9% as the enzyme is saturated with fructose 1,6-bisphosphate under these conditions. The change in protein fluorescence as the enzyme is saturated also is shown in Figure 3; this change is about 90% complete when the enzyme is only about 50% saturated. The polarization of the enzyme fluorescence changes only slightly when the enzyme is saturated with fructose 1,6-bisphosphate. As expected from the quenching data, the protein fluorescence lifetime

Table III: Parameters for Fructose 1,6-Bisphosphate and Fructose 6-Phosphate Binding to Phosphofructokinase in 5 mM Citrate, pH 7.0.[a]

Ligand	Type of Data	Enzyme (mg/ml)	K_A (μM^{-1})	K_A' (μM^{-1})	K_B (μM^{-1})	K_P (μM^{-1})
Fru-1,6-P_2	Binding	0.15	0.967	1.46	0.566	0.0051
[Fru-1,6-P_2[b]	Binding	0.15	0.651	2.06	0.485	0.016 (fixed)]
Fru-1,6-P_2	Fluorescence	0.15	0.943	2.28	0.608	0.018
Fru-1,6-P_2	Activity	0.15	0.620	2.39	1.01	0.014
Fru-1,6-P_2	Stokes' radii	0.15	0.493	2.66	1.09	0.021
Fru-6-P	Binding	0.75	0.117	1.99	0.088	0.032
Fru-6-P	Fluorescence	0.75	0.122	1.71	0.122	0.017
Fru-6-P	Activity	0.75	0.260	1.66	0.092	0.010

[a] The data in Figures 2D, 3, and 4 were fit to eq A2-A5 describing mechanism II. All experiments were carried out in 0.1 M Tris-Cl (pH 7.0), 5 mM dithiothreitol, and 1 mM ethylenediaminetetraacetic acid, and 5 mM citrate, 5°. [b] The value of K_P was assumed to be 0.016 μM^{-1} (an average value from other measurements) in fitting the data.

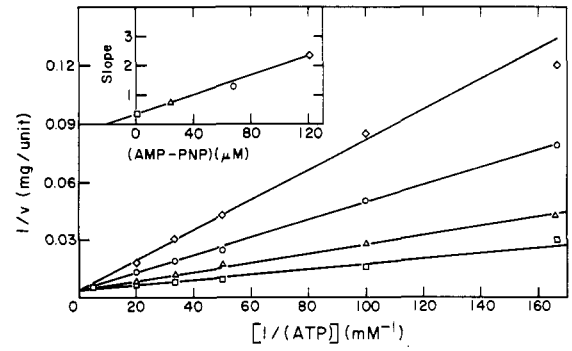


FIGURE 5: A plot of the reciprocal steady-state initial velocity *vs.* the reciprocal ATP concentration at several concentrations of AMP-PNP (pH 8.0). The AMP-PNP concentrations were 0 (□), 24.3 (△), 60.8 (○), and 122 (◇) μM. The assays were carried out as described in the Experimental Section at 23° and the ATP concentrations were varied between 6 and 50 μM. The fructose 6-phosphate concentration was 2 mM. The inset is a plot of the slopes from a linear least-squares fit of the individual lines *vs.* the AMP-PNP concentration.

also decreases significantly when the enzyme is saturated with ligand (unpublished results).

Similar results to those described above were found for the binding of fructose 6-phosphate to enzyme in 5 mM citrate (pH 7.0). The binding isotherm for the binding of fructose 6-phosphate to 0.75 mg of phosphofructokinase/ml is shown in Figure 2D. It was necessary to use a higher protein concentration than in the fructose 1,6-bisphosphate binding studies because of the weaker binding of fructose 6-phosphate to the enzyme. However, the Stokes' radius of the enzyme with no fructose 6-phosphate present is 49 Å (0.1 M Tris-Cl (pH 7.0), 5 mM citrate, 5 mM dithiothreitol, and 1 mM ethylenediaminetetraacetic acid) indicating a substantial amount of dimer is present. The Stokes' radius at 0.1–10 mM fructose 6-phosphate, 0.15 mg of phosphofructokinase/ml, 0.1 M Tris-Cl (pH 7.0), 5 mM dithiothreitol, and 1 mM ethylenediaminetetraacetic acid is 66 Å (Lad *et al.*, 1973) showing that the enzyme polymerizes as it is saturated with fructose 6-phosphate. A complete investigation of the change in Stokes' radius with increasing fructose 6-phosphate was not carried out because of the large quantities of protein required. However, a measure of the change in the aggregation state of the enzyme may be obtained by determining the change in the specific activity of the enzyme when it is incubated with various concentrations of fructose 6-phosphate. These measurements were done similarly to those described above for fructose 1,6-bisphosphate. The changes in specific activity are illustrated in Figure 4. The protein fluorescence of phosphofructokinase is again quenched, about 5.8%, as the enzyme is saturated with fructose 6-phosphate as shown in Figure 4, and again the change in protein fluorescence appears to be 90% complete when the enzyme is only about 50% saturated with ligand.

The binding isotherms and changes in specific activity, Stokes' radius, and protein fluorescence with varying ligand concentration are very similar in nature for fructose 6-phosphate and fructose 1,6-bisphosphate (compare Figures 3 and 4). All of the data of Figures 2D, 3, and 4 were fit to the mechanism represented by II. The function r is given by eq A2, assuming one binding site per subunit of molecular weight 80,000. The observed changes in specific activity were assumed to be due to the formation of tetramer, and only the tetrameric species was assumed to be active (Parmeggiani *et al.*, 1966; Lad *et al.*, 1973). Therefore, the initial velocity can be written in terms of the fraction of protein in the tetrameric state, as in eq A3. The average Stokes' radius was expressed in terms of the dimer and tetramer Stokes' radii according to eq A4. The Stokes' radius of the dimer was taken as 43 Å and that of the tetramer as 68 Å (Pavelich and Hammes, 1973). For the protein fluorescence quenching data, it was assumed that only ligand binding to the tighter site on both the dimer and tetramer resulted in a fluorescence change. The later assumption is justified by the observation that the fluorescence changes seem to be associated with saturation of the first type of site (Figures 3 and 4); therefore the per cent change in fluorescence can be expressed by eq A5. The equilibrium constants obtained from the non-linear least-squares computer fits to the data are given in Table II, and the solid lines in Figures 2D, 3, and 4 were calculated using eq A3–A5 and the parameters in Table III. The average relative parameter deviation ranged from 4 to 58%. The variation of citrate concentration from 0.5 to 5 mM did not alter the binding of fructose 1,6-bisphosphate to the enzyme within the experimental uncertainties.

Inhibition of Phosphofructokinase Activity by Adenylyl Imidodiphosphate at pH 8.0. The inhibition of the steady-state initial velocity of phosphofructokinase by the ATP analog, adenylyl imidodiphosphate (AMP-PNP) (Yount *et al.*, 1971), at pH 8.0 is shown in Figure 5 where the reciprocal initial velocity is plotted *vs.* the reciprocal ATP concentration at several different concentrations of AMP-PNP. The inhibition is strictly competitive with respect to ATP as shown by the inset plot of slopes *vs.* inhibitor concentration. From a linear least-squares analysis of the data, the inhibition constant for AMP-PNP was determined to be 21.5 μM while the Michaelis constant for ATP was found to be 20.4 μM. The β,γ-methyleneadenosine 5′-triphosphate also inhibited phosphofructokinase in a competitive manner but had a much higher inhibition constant, 1.9 mM. An inhibition study at pH 7.0 was not attempted because of the com-

plex inhibition of the catalysis by ATP and the sigmoidal initial velocity–fructose 6-phosphate isotherms.

Effect of AMP-PNP on Fructose 6-Phosphate Binding. The binding of fructose 6-phosphate to 0.84 mg of phosphofructokinase/ml in the presence of 0.22 mM AMP-PNP, 2.5 mM potassium phosphate, and 4.5 mM $MgCl_2$ at pH 7.0 and 8.0 is shown in Figure 6. The binding isotherm at pH 8.0 is hyperbolic; however, at pH 7.0 the binding isotherm is sigmoidal with a Hill coefficient of 2.8. A high protein concentration was required in these experiments because the AMP-PNP greatly depresses the binding. The Stokes' radius of the enzyme was not determined but it may reasonably be extrapolated that the enzyme is at least tetrameric and larger aggregates probably also are present (Pavelich and Hammes, 1973; Lad *et al.*, 1973). If the binding constant for the AMP-PNP–enzyme interaction at pH 7.0 is at all comparable to the inhibition constant at pH 8.0, the enzyme should be essentially saturated with AMP-PNP (0.22 mM) under the conditions used to obtain the binding isotherm at pH 7. Control experiments showed that phosphofructokinase (0.15 mg/ml) did not hydrolyze AMP-PNP significantly (<2%) when incubated 24 hr in the presence of fructose 6-phosphate and $MgCl_2$ at pH 7.0 or 8.0.

The data at pH 7.0 in Figure 6 were fit to the sequential mechanism shown in III, which is analogous to mechanism

III

I and II in concept. The expression for r is given by eq A6. The least-squares fit of the data is shown as the solid line in Figure 6 and the values of the association constants are $K_{S1} = 3.61 \times 10^{-3}\ \mu M^{-1}$ and $K_{S2} = 1.75 \times 10^{-1}\ \mu M^{-1}$. The data also could be fit by a concerted mechanism (Monod *et al.*, 1965) assuming a cooperative unit of four binding sites. The data at pH 8.0 in Figure 6 were fit to eq 2 and the association constant obtained is $K_S = 28.2\ mM^{-1}$. The apparent Michaelis constant for fructose 6-phosphate in the presence of 0.22 mM ATP, 4.5 mM $MgCl_2$, and 2.5 mM potassium phosphate (pH 8.0) is 49.8 μM.

$$r = K_S(S)/[1 + K_S(S)] \qquad (2)$$

Discussion

Rabbit muscle phosphofructokinase consists of essentially identical polypeptide chains (Pavelich and Hammes, 1973; Aaronson and Frieden, 1972; Paetkau *et al.*, 1968) which undergo polymerization reactions that are concentration and pH dependent (*cf.* Aaronson and Frieden, 1972; Hofer, 1973; Pavelich and Hammes, 1973; Lad *et al.*, 1973). The fundamental molecular unit for polymerization appears to be a dimer (Hofer, 1973; Lad and Hammes, 1974). The binding of fructose phosphates also can be understood in terms of enzyme dimer units. The models used to analyze the binding data (I–III) contain the minimal number of parameters needed to describe the binding data quantitatively. These models are probably an oversimplification in that protein aggregates other than the dimer and tetramer are probably present, especially at the higher protein concentrations used. Moreover, these models are not unique and more general models also will satisfactorily describe the data (*cf.* Koshland, 1969). However, any model must produce a decreasing enzyme affinity for the fructose phosphates as the degree of enzyme saturation increases. At the same time, the binding of ligands must convert the

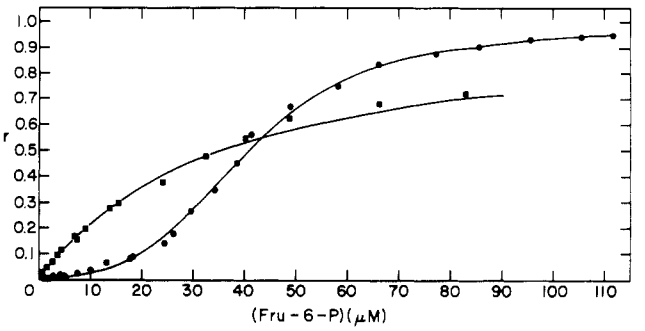


FIGURE 6: A plot of the moles of fructose 6-phosphate bound per mole of enzyme (molecular weight 80,000), r, *vs.* the concentration of free fructose 6-phosphate (Fru-6-P), in the presence of 0.22 mM AMP-PNP at 5°, pH 7.0 (●) (25 mM imidazole-Cl, 5 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid, 4.5 mM $MgCl_2$, and 2.5 mM potassium phosphate) and pH 8.0 (■) (25 mM glycylglycine, 5 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid, 4.5 mM $MgCl_2$, and 2.5 mM potassium phosphate). The concentration of enzyme was 0.84 mg/ml. The solid lines were calculated with eq A6 for data at pH 7.0 or eq 2 for data at pH 8.0 and the parameters given in the text.

dimer to tetramer (Figures 3 and 4). The simplest type of mechanism consistent with these findings is the assumption of negative cooperativity in binding to both dimer and tetramer, with the negative cooperativity being less in the tetramer than in the dimer, thus producing a conversion of dimer to tetramer as the enzyme is saturated with ligand. The dimer is a logical fundamental unit in the binding mechanism, but it is not required by the data: for example, four different binding constants associated with binding to the tetramer also would describe the data satisfactorily. In the presence of AMP-PNP, the positive cooperativity can be explained in terms of either a sequential or a concerted mechanism, but a sequential mechanism appears to be more appropriate since it parallels the mechanisms utilized to explain the negative cooperativity. In all of the mechanisms, the assumption that different types of binding sites preexist is equivalent to assuming that conformational changes accompany binding and produce cooperativity. Equilibrium binding studies cannot differentiate between these two assumptions. Because of the experimental uncertainties and simple models used, the parameters in Tables I–III are probably not more reliable than a factor of 2.

The binding of fructose phosphates to the tetrameric enzyme at pH 8.0 (0.15 mg of phosphofructokinase/ml) indicates at least two different conformational states of the subunits can exist. The degree of negative cooperativity in the binding process for both fructose 6-phosphate and fructose 1,6-bisphosphate is similar at pH 8.0 as $K_{S1}/K_{S2} \approx 60$ for both ligands, although fructose 1,6-bisphosphate binds four times more tightly to the enzyme. The negative cooperativity observed is similar to the results obtained by Setlow and Mansour (1972) for the binding of fructose 6-phosphate and fructose 1,6-bisphosphate to sheep heart phosphofructokinase. However, they found two binding sites per 100,000 g-atoms of protein, whereas the data presented here indicate one site per 80,000 g-atoms of protein under very similar conditions. Approximately one site per 90,000 g-atoms also was reported for the rabbit muscle enzyme by Kemp and Krebs (1967) at higher enzyme concentrations.

At protein concentrations where higher aggregates than tetramer predominate (0.75 mg/ml of phosphofructokinase, pH 8.0), the binding data indicate no cooperativity occurs (Figure 1C). Actually a close examination of Figure 1C suggests a small amount of negative cooperativity may still

exist, but this may be due to the presence of some tetrameric enzyme. Apparently aggregation of the enzyme constrains the subunits so as to reduce the interactions between fructose phosphate binding sites.

The binding of the fructose phosphates to the enzyme at pH 7 in the presence of phosphate is qualitatively similar to that at pH 8 (Figure 2). However, at a protein concentration of 0.15 mg/ml, the enzyme is a mixture of dimer and tetramer (R_S = 55 Å) which polymerizes to tetramer as it is saturated with ligand. The specific activity of the enzyme increases from 60 to 128 units/mg on saturation. Mechanism II satisfactorily accounts for the binding data. Both ligands bind somewhat tighter to the strong binding sites of the tetramer than to the dimer. However, the degree of negative cooperativity is much more pronounced for fructose 1,6-bisphosphate than for fructose 6-phosphate (Table II) since the bisphosphate binds nearly an order of magnitude more tightly at the strong binding site of the dimer and tetramer than at the weak binding site of the tetramer. The degree of negative cooperativity for fructose phosphate binding also becomes less pronounced as either the potassium phosphate or the enzyme concentration is increased. Either variation has the effect of lowering K_A and K_A' whereas K_B remains relatively constant. These results, together with the results obtained at 0.75 mg of phosphofructokinase/ml (pH 8.0) probably account for the hyperbolic fructose 6-phosphate binding isotherms observed by Kemp and Krebs (1967) at high protein concentration in α-glycerol phosphate (pH 7.0). If phosphate is assumed to be a competitive inhibitor of fructose phosphate binding, inhibition constants of 0.28, 0.30, and 0.34 mM^{-1} can be estimated for the dimer, "tight" tetramer, and "loose" tetramer sites, respectively, in the case of fructose 1,6-bisphosphate and 0.17, 0.22, and 0.086 mM^{-1} in the case of fructose 6-phosphate. These constants are quite imprecise since they are based on only a few phosphate concentrations. Except for the "loose" tetramer site, the constants are in reasonable agreement and are slightly smaller than the inhibition constants found at pH 8.0. The rather high value for the "loose" tetramer site in the presence of fructose 1,6-bisphosphate can be attributed to the imprecision in the binding constants at the different phosphate concentrations. The phosphate independent values of K_A, K_A', and K_B are 16.2, 37.5, and 0.839 μM^{-1} for fructose 1,6-bisphosphate and 1.48, 1.95 and 0.495 μM^{-1} for fructose 6-phosphate. Fructose 6-phosphate appears to bind much more weakly to the dimer and "tight" tetramer sites at pH 7.0 than at pH 8.0. The fructose 1,6-bisphosphate binding parameters at pH 7.0 and 8.0 are similar.

The competition of phosphate for the fructose phosphate sites at both pH 7.0 and 8.0 undoubtedly is responsible for the stability of phosphofructokinase in concentrated phosphate buffers. The enzyme is irreversibly denatured under conditions of low protein concentration ($\leq$0.15 mg/ml) and low occupancy of the phosphate binding sites on the enzyme. However, saturating concentrations of citrate stabilize the dimer somewhat in the absence of other ligands (Lad *et al.*, 1973).

At pH 7 in the presence of 5 mM citrate the enzyme is essentially dimeric with a Stokes' radius of 37–43 Å. The binding isotherms (Figure 2D) are qualitatively similar to those observed in phosphate. The saturation of enzyme by ligands is paralleled by an increase in enzymic activity and the Stokes' radius of the enzyme and by a quenching of protein fluorescence (Figures 3 and 4). The binding, activity, molecular size, and fluorescence data can be quantitatively described by mechanism II, and the parameters obtained by independently fitting each set of data agree within a factor of about 2, which is quite satisfactory. The monomer–dimer equilibrium constant, K_P, is similar in all cases except in the fitting of the fructose 1,6-bisphosphate binding curve, where it is somewhat lower than the other values. If the value of K_P is fixed at 0.016 μM^{-1}, an average value from the other experiments, a very good fit of the binding data for fructose 1,6-bisphosphate is obtained, and the parameters obtained with this assumption are included in Table III. The observed quenching of protein fluorescence, which appears to be mainly associated with the binding of ligands to the tight site, strongly suggests a conformational change accompanies ligand binding but cannot be entirely separated from the possibility of fluorescence changes due to polymerization.

Citrate significantly weakens the binding of the fructose phosphates to the enzyme. While part of this effect may be due to stabilization of the dimer relative to the tetramer, the inhibition is more complex than this as all of the binding constants are decreased, while K_P does not differ greatly from the values obtained at low phosphate concentrations (Tables II and III). The binding of the fructose phosphates was not significantly altered over a tenfold range in citrate concentration (0.5–5 mM) so that citrate inhibition of binding is clearly not competitive in nature. The degree of negative cooperativity in 5 mM citrate was considerably less than in phosphate at pH 8.0 for both ligands. The physiological mechanism for citrate inhibition is still unclear: this work indicates that citrate may weaken the binding of fructose 6-phosphate to the enzyme, while previous work (Lad *et al.*, 1973) indicates citrate depolymerizes the enzyme to inactive aggregates at pH 7. Both of these phenomena may be of importance for regulation, but the former appears to represent a "fine tuning" type of regulation whereas the latter would provide a larger and more dramatic metabolic effect. The citrate inhibition may be overcome at saturating concentrations of the fructose phosphates by polymerizing the inactive dimer to fully active tetramer (Figures 3 and 4).

The sigmoidal binding isotherm for fructose 6-phosphate binding to the enzyme at pH 7.0 in the presence of the ATP analog AMP-PNP is similar to that observed in steady-state kinetic studies. As expected, no cooperativity in the binding process is observed at pH 8.0 in the presence of AMP-PNP. The enzyme consists of tetramer and higher aggregates under the conditions of the binding experiments. Thus the positive cooperativity in fructose 6-phosphate binding to the enzyme undoubtedly is due to subunit interactions rather than to protein polymerization. Both sequential and concerted models fit the data adequately. The degree of cooperativity in the binding, as judged by the Hill coefficient is high. Although AMP-PNP induces positive cooperativity in the binding of fructose 6-phosphate, the strength of the binding is considerably reduced relative to that observed at millimolar phosphate and citrate. The binding constant at pH 8 is nearly equal to the reciprocal of the free ligand concentration at 50% saturation at pH 7.0 (24.4 mM^{-1}). The apparent Michaelis constant for fructose 6-phosphate at pH 8.0 is slightly higher than the reciprocal of the binding constant which is not unreasonable since the Michaelis constant is a complex function of rate constants and ATP and AMP-PNP may not be exactly equivalent.

Fructose 6-phosphate and fructose 1,6-bisphosphate are

substrates of the enzyme and also activate the enzyme by relieving the MgATP inhibition. Yet only a single binding site per subunit is found in the binding studies reported here. The possibility exists that a single active site and a single regulatory site each require two subunits, but this seems unlikely since all of the fructose phosphate binding sites appear to be identical in protein aggregates larger than the tetramer and the Hill coefficient in the presence of AMP-PNP is greater than two. Evidently occupation of all of the catalytic sites by fructose phosphates (and other phosphates) is sufficient to eliminate MgATP binding to the inhibitory site. A more detailed study of the mechanism of MgATP inhibition is currently being carried out.

In summary, the results obtained indicate that the binding of fructose 6-phosphate and fructose 1,6-bisphosphate to phosphofructokinase can exhibit negative, positive, or no cooperativity under different experimental conditions. A sigmoidal fructose 6-phosphate binding isotherm is found in the presence of AMP-PNP, indicating altered homotropic interactions are induced by the binding of the nucleotide. Polymerization of the enzyme has been shown not to be directly involved in inducing this positive cooperativity; however polymerization probably is responsible for the relief of citrate inhibition by the fructose phosphates. Although the detailed regulatory mechanism for phosphofructokinase remains to be elucidated, it seems likely that homotropic interactions, heterotropic interactions, and protein polymerization all play a role.

Appendix

The moles of ligand bound per mole of monomeric enzyme (molecular weight 80,000), r, for mechanism I can be derived from the table in Chart I and is given by

$$r = [(X_1) + 2(X_3) + (X_5) + 3(X_6) + 2(X_7) + (X_8) + 2(X_{10}) + (X_{11}) + 4(X_{12}) + 3(X_{13}) + 2(X_{14}) + 2(X_{15}) + (X_{16}) + 3(X_{18}) + 2(X_{19}) + (X_{20})]/4\sum_{i=0}^{20}(X_i) \quad \text{(A1)}$$

The moles of ligand bound per mole of monomeric enzyme (molecular weight 80,000), r, for mechanism II can be derived from the table in Chart II and is given by

$$r = \frac{(X_1) + (X_3) + 2(X_4) + 3(X_5) + 4(X_6)}{[2(X_0) + 2(X_1) + 4(X_2) + 4(X_3) + 4(X_4) + 4(X_5) + 4(X_6)]} \quad \text{(A2)}$$

If only the tetrameric enzyme is assumed to be active, the fractional activity is given by eq A3 where A_x is the activity of the sample, A_∞ is the activity of the tetramer, and A_0 is the activity of the dimer at pH 8.0.

$$\frac{A_x - A_0}{A_\infty - A_0} = \frac{4[(X_2) + (X_3) + (X_4) + (X_5) + (X_6)]}{[2(X_0) + 2(X_1) + 4(X_2) + 4(X_3) + 4(X_4) + 4(X_5) + 4(X_6)]} \quad \text{(A3)}$$

The Stokes' radius, R_S^x, for the enzyme according to mechanism II is given by eq A4 where R_2 = 43 Å (Stokes' radius of dimer), R_4 = 68 Å (Stokes' radius of tetramer), $M_2 = 1.6 \times 10^5$ (molecular weight of dimer), $M_4 = 3.2 \times 10^5$ (molecular weight of tetramer), $P_2 = (X_0) + (X_1)$ (dimer concentration), and $P_4 = (X_2) + (X_3) + (X_4) + (X_5) + (X_6)$ (tetramer concentration).

$$R_S^x = \frac{R_2P_2M_2 + R_4P_4M_4}{P_2M_2 + P_4M_4} \quad \text{(A4)}$$

CHART I
Mechanism I

Molecular Species	Species Symbol	Species Concentration
	X_0	(X_0)
	X_1	$2K_{S1}(X_0)(S)$
	X_2	$2K_{I1}(X_0)(I)$
	X_3	$K_{S1}^2(X_0)(S)^2$
	X_4	$K_{I1}^2(X_0)(I)^2$
	X_5	$2K_{S1}K_{I1}(X_0)(S)(I)$
	X_6	$2K_{S2}K_{S1}(X_0)(S)^3$
	X_7	$2K_{I2}K_{S2}^2(X_0)(S)^2(I)$
	X_8	$2K_{S2}K_{I1}^2(X_0)(S)(I)^2$
	X_9	$2K_{I2}K_{I1}(X_0)(I)^3$
	X_{10}	$4K_{S2}K_{S1}K_{I1}(X_0)(S)^2(I)$
	X_{11}	$4K_{I2}K_{S1}K_{I1}(X_0)(S)(I)^2$
	X_{12}	$K_{S2}^2K_{S1}^2(X_0)(S)^4$
	X_{13}	$2K_{I2}K_{S2}K_{S1}^2(X_0)(S)^3(I)$
	X_{14}	$K_{I2}^2K_{S1}^2(X_0)(S)^2(I)^2$
	X_{15}	$K_{S2}^2K_{I1}^2(X_0)(S)^2(I)^2$
	X_{16}	$2K_{S2}K_{I2}K_{I1}^2(X_0)(S)(I)^3$
	X_{17}	$K_{I2}^2K_{I1}^2(X_0)(I)^4$
	X_{18}	$2K_{S2}^2K_{S1}K_{I1}(X_0)(S)^3(I)$
	X_{19}	$4K_{S2}K_{I2}K_{S1}K_{I1}(S)^2(I)^2$
	X_{20}	$2K_{I2}^2K_{S1}K_{I1}(X_0)(S)(I)^3$

CHART II
Mechanism II

Molecular Species	Species Symbol	Species Concentration
	X_0	(X_0)
	X_1	$K_A(X_0)(S)$
	X_2	$K_P(X_0)^2$
	X_3	$2K_A'K_P(X_0)^2(S)$
	X_4	$K_A'^2K_P(X_0)^2(S)^2$
	X_5	$2K_BK_A'^2K_P(X_0)^2(S)^3$
	X_6	$K_B^2K_A'^2K_P(X_0)^2(S)^4$

The fractional change in fluorescence accompanying ligand binding for mechanism II is given by eq A5 assuming only binding to the tight sites (squares) produces a fluorescence change. In this equation F_0 is the fluorescence of the unliganded enzyme, F_∞ is the fluorescence of the enzyme with all tight sites occupied, and F_x is the fluorescence of the sample under consideration.

$$\frac{F_0 - F_x}{F_0 - F_\infty} = \frac{2[(X_1) + (X_3) + 2(X_4) + 2(X_5) + 2(X_6)]}{[2(X_0) + 2(X_1) + 4(X_2) + 4(X_3) + 4(X_4) + 4(X_5) + 4(X_6)]} \quad \text{(A5)}$$

The concentration of X_0 was computed by solving the following quadratic equation derived from mass balance equations:

$$(X_0)^2\{4K_p[1 + 2K_a'(S) + K_a'^2(S)^2 + 2K_BK_A'^2(S)^3 + K_B^2K_A'^2(S)^4]\} + (X_0)[2 + 2K_a(S)] - (E)_T = 0$$

The total enzyme concentration, $(E)_T$, is expressed as the monomer concentration, with a molecular weight of 80,000.

CHART III
Mechanism III

Molecular Species	Species Symbol	Species Concentration
	X_0	(X_0)
	X_1	$2K_{S1}(X_0)(S)$
	X_2	$K_{S1}^2(X_0)(S)^2$
	X_3	$2K_{S2}K_{S1}^2(X_0)(S)^3$
	X_4	$K_{S2}^2K_{S1}^2(X_0)(S)^4$

$$r = \frac{(X_1) + 2(X_2) + 3(X_3) + 4(X_4)}{4[(X_0) + (X_1) + (X_2) + (X_3) + (X_4)]} \quad \text{(A6)}$$

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