Rat Liver Phosphofructokinase: Kinetic Activity under Near-Physiological Conditions†

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ABSTRACT: Rat liver phosphofructokinase (PFK) has been purified to near homogeneity by the combination and modification of two existing procedures. The kinetic properties of the purified enzyme have been studied under the near-physiological conditions of pH 7.0, 120 mM KCl, and 3 mM MgATP. Assays performed under these conditions are generally nonlinear with time; the shape of the progress curves are influenced by the order of addition of substrates to the reaction mixture. High initial velocities, which decelerate with time, are obtained after initiation with Mg^{2+}, or ATP, whereas accelerating low initial rates occur in assays begun by the addition of fructose 6-phosphate (F6P). The steady-state activity of rat liver PFK, attained after the completion of either type of transient, is characterized by an extremely low affinity and high degree of positive cooperativity toward varying F6P concentration (K_m = 6 mM; Hill coefficient > 4). The primary mode of action of the physiological activators (AMP, fructose 1,6-bisphosphate, and inorganic phosphate) and inhibitors (MgATP, citrate, and H^+) studied is to alter the K_m for F6P, with little or no effect on V_{max}. The simultaneous action of the activators at physiologically optimal concentrations produces a synergistic response which mitigates MgATP inhibition to a large extent. A substantial decrease in MgATP inhibition is also found to be necessary to explain the affinity for F6P that PFK must demonstrate in vivo given the low levels of F6P which are found in the cell.

Phosphofructokinase (EC 2.7.1.11) is an important regulatory enzyme of carbohydrate metabolism and as such has been the subject of intensive investigation [for a recent review, see Uyeda (1979)]. The majority of this work has been performed on the enzyme isolated from primarily glycolytic cells, most notably rabbit muscle. Carbohydrate metabolism of cells capable of performing gluconeogenesis, such as liver and kidney, is fundamentally more complex, and the PFK[1] isolated from these sources has been found to have, as expected, altered characteristics. Liver isozymes of PFK from a variety of species including rabbit (Kemp, 1971; Ramaiah & Tejwani, 1973), pig (Massey & Deal, 1973), chicken (Kono & Uyeda, 1974), sheep (Brock, 1969), and rat (Brand & Söling, 1974; Dunaway & Weber, 1974; Underwood & Newsholme, 1965) have been studied. Since a large majority of the studies of gluconeogenesis, including those in our own laboratory, are performed with the rat, we were interested in more closely investigating the kinetic properties of the liver enzyme from this source.

Underwood & Newsholme (1965) have briefly described some allosteric properties of a partially purified preparation of rat liver PFK. More recently, Brand & Söling (1974) have purified rat liver PFK and examined its kinetics under conditions of very low nucleotide concentration at pH 8, where the enzyme did not exhibit any allosterism. With this approach, they were able to determine the true Michaelis and inhibition constants as well as the kinetic mechanism by performing initial velocity and product inhibition experiments and interpreting these data in the conventional manner. Under the more physiological conditions of pH 7 and high nucleotide concentration, however, the allosteric nature of the enzyme is highly evident and the kinetic properties are appreciably altered. Indeed, the allosteric influences of various cellular metabolites on the kinetic behavior probably have at least as great a role in the ultimate determination of the enzyme's activity in the cell as do the nonallosteric kinetic constants. Consequently, we present here a more detailed examination of the allosteric properties of the purified rat liver PFK, with particular attention focused upon the enzyme behavior under conditions similar to those likely to be found in the cell. The results of this study indicate that the kinetic activity of the isolated enzyme at cellular concentrations of MgATP (3 mM), KCl (120 mM), and F6P (60 μM) at 37 °C and pH 7.0 is insufficient to account for the necessary cellular activity unless the combined action of the several relevant effectors such as FBP, AMP, and P_i is considered. Even then the PFK activity realized is a small percentage of the total available. This low activity is a consequence of a high K_m for F6P and a very high degree of cooperativity and not of an effect on the maximal enzyme activity, on which the allosteric ligands have little effect.

In addition, a purification procedure is described that incorporates some of the attributes of the two purifications previously published (Dunaway & Weber, 1974; Brand & Söling, 1974), resulting in a more rapid and higher yielding purification. A preliminary report of some of these results has been presented (Reinhart, 1977).

Materials and Methods

Reagents and enzymes were from the following sources: ATP (sodium salt) and NADH, P-L Biochemicals; F6P and FBP (sodium salts), DTT, PEP (monopotassium salt), and Mops, Sigma Chemical Co.; aldolase, triosephosphate isomerase and glycerol-3-phosphate dehydrogenase (ammonium sulfate suspensions) and lactate dehydrogenase and pyruvate kinase (glycerol solutions), Boehringer Mannheim; chromatography resins, Whatman (DE-32) and Pharmacia (Sepharose 2B). Glycerol-3-phosphate dehydrogenase and triosephosphate isomerase were generally obtained as the mixed

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[1] Abbreviations used: PFK, phosphofructokinase; F6P, fructose 6-phosphate; DTT, dithiothreitol; Mops, 4-morpholinepropanesulfonic acid; EDTA, disodium ethylenediaminetetraacetate; PEP, phosphoenolpyruvate; FBP, fructose 1,6-bisphosphate; P_i, inorganic phosphate; NaDodSO$_4$, sodium dodecyl sulfate; Cl_3AcOH, trichloroacetic acid.

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suspension in the ratio of 10:1, respectively (weight basis). Other chemicals and reagents were of the highest purity available. Deionized, distilled water was used throughout.

Kinetic assays were performed on a single-beam spectrophotometer consisting of a Beckman DU monochromator fitted with a Gilford Model 252 lamp housing and photomultiplier electronics and a Hewlett-Packard 7101B strip chart recorder. Digital data acquisition for the nonlinear regression analysis described below was performed by interfacing a Hewlett-Packard 9825A desk top computer to the BCD output of the Gilford and coupling the data transfer to a line frequency based real-time clock as described previously (Reinhart, 1979).

Optimal enzyme activity assays were performed in the following manner. In a final volume of 1.0 mL the reaction mixture contained 50 mM Tris-HCl, 100 mM KCl, 20 mM $(NH_4)_2SO_4$, 2 mM DTT, 3 mM $MgCl_2$, 1 mM ATP, 1 mM F6P, 0.2 mM NADH, 100 μM EDTA, pH 8.0, 200 μg of aldolase, and 20 μg of triosephosphate isomerase–glycerol-3-phosphate dehydrogenase. Assays were performed at 25 °C, initiated by the addition of PFK, and the linear rate, usually attained after a short lag, was directly measured. Assays performed during the PFK purification included 1 mM AMP to inhibit FBPase.

Allosteric kinetic properties of rat liver PFK were investigated in assay mixtures containing 50 mM Mops–KOH, 100 mM KCl, 100 μM EDTA, 2 mM DTT, 0.2 mM NADH, 400 μg of aldolase, and 40 μg of triosephosphate isomerase–glycerol-3-phosphate dehydrogenase, pH 7.0, in a final volume of 1.0 mL. Most assays were performed at a fixed concentration of 3 mM ATP with a total $MgCl_2$ concentration of 5 mM. In experiments where ATP concentration was varied, total $MgCl_2$ was always kept in 2 mM excess of the ATP. F6P was varied as indicated for each experiment. Stock solutions of F6P and ATP were assayed enzymatically and kept frozen between experiments. Purified liver PFK concentration was 0.1 μg/mL in the assay unless otherwise noted. It was usually added in a volume of 10 μL of storage buffer which contributed 0.2 mM P_i, 0.2% glycerol, 10 μM F6P, and 30 μM $MgSO_4$ to the assay. The effect of this contribution was minor.

Assays in which the effect of FBP was examined were performed with pyruvate kinase (40 μg/mL) and lactate dehydrogenase (40 μg/mL) as coupling enzymes and included 1 mM PEP. A smaller amount of PFK (0.04 μg/mL) was assayed, and the extent of reaction was limited to the generation of an additional 10 μM FBP. Assays using the aldolase couple were initiated by the addition of ATP whereas those utilizing the pyruvate kinase couple were begun by the addition of PFK. The temperature of all assays was maintained at 25 °C unless otherwise noted.

Coupling enzymes in all cases were desalted prior to use by employing the centrifuge column procedure of Orly & Selinger as described by Penefsky (1977). PFK activity is presented as units per milligram of protein where 1 unit is equal to the production of 1 μmol of FBP (or ADP) per min.

Most assays performed at pH 7 were nonlinear with respect to time, and the curves were fit to the general expression describing first-order kinetic transients as described by Reinhart (1980). Digitized (time; absorbance) data were collected during the course of an assay at 1-s intervals beginning 10 s after initiation of the assay. Assays were terminated either after 10 min or when the substrate concentration had decreased by 20 μM, whichever occurred first. For the pyruvate kinase coupled assays, this latter limit was set at 10 μM. Sufficiently low amounts of PFK were assayed so that assays lasted at least 2–3 min at maximum rates. Since most assays were performed with millimolar concentrations of substrates, it was assumed that these extents of reaction would have a negligible effect on the assays and hence they could be evaluated assuming constant substrate concentration [see Reinhart (1980)].

Sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis was performed as described by Weber & Osborn (1969). Protein samples were denatured by incubating in a boiling water bath for 10 min in the presence of 1% $NaDodSO_4$, 10% glycerol, 10 mM NaP_i (pH 7.0), and 1% DTT. The samples were then layered on 7.5% polyacrylamide gels, and migration (at 7–8 mA/tube) was followed with bromphenol blue as a marker. Gels were stained for protein with 0.25% coomassie blue R in a 50% methanol–5% acetic acid solution for 3–6 h and then destained following the procedure of Greaser & Gergely (1971). Molecular weight determination was performed by comparing the mobility of PFK to the mobility of the following standards according to the procedure of Weber & Osborn (1969): phosphorylase *a* (94000), bovine serum albumin (68000), ovalbumin (43000), lactate dehydrogenase (36000), α-chymotrypsinogen (25700) and β-lactoglobulin (18400). Gels were scanned by using a Gilford Model 2410-S linear transport. Absorbance at 546 nm was measured with a Beckman DU monochromator adapted with a Gilford 2220 conversion unit connected to a Gilford 6040 strip chart recorder.

Protein concentrations were determined by using the method of Lowry et al. (1951) with gravimetrically prepared bovine serum albumin fraction V as a standard. Samples and standards were precipitated in 5% Cl_3AcOH and washed once with 3% Cl_3AcOH to remove compounds which might interfere with the colorimetric reaction.

Hill coefficients were obtained from the least-squares linear regression of the data plotted as log $[(V_{max} - v)/v]$ vs. log [F6P] where v is the steady-state rate and V_{max} is the rate at saturating F6P. V_{max} was estimated from the plots of v vs. [F6P] shown. Only those rates which fell between 10 and 90% of V_{max} were used in the Hill coefficient calculation.

Purification. The procedure of Dunaway & Weber (1974) for purifying rat liver phosphofructokinase did not yield homogeneous enzyme in our hands; that of Brand & Söling (1974) is somewhat time consuming. We have purified rat liver PFK to near homogeneity by combining the initial steps of the Dunaway procedure with the ion-exchange and gel filtration steps of the Brand procedure, with some modification. The procedure can be performed easily in 3 days and includes only a heat step, ammonium sulfate precipitation, and DEAE ion-exchange and gel filtration chromatography.

(*1*) *Homogenization.* Fresh livers from fed, male Sprague–Dawley rats of mixed age are homogenized in either a Potter-Elvehjem homogenizer or a Waring blender with 3 volumes of chilled buffer containing 50 mM Tris-HCl, 50 mM NaF, 5 mM DTT, and 1 mM ATP, pH 8.0. The homogenate is then spun at 100000*g* for 60 min and the resulting supernatant solution is poured through glass wool.

(*2*) *Heat Treatment.* The solution is brought to 60 °C in a boiling-water bath with continuous stirring. The suspension is kept at 60 ± 1 °C for 3 min and then cooled to less than 10 °C by swirling in an ice–salt bath. After centrifugation for 30 min at 20000*g*, the supernatant solution is once again decanted through glass wool.

(*3*) *Ammonium Sulfate Precipitation.* A total of 18.5 g of enzyme-grade ammonium sulfate per 100 mL of solution is added slowly. Once the salt is completely dissolved, the mixture is kept without stirring at 4 °C for 90 min and then

Table I: Summary of Rat Liver PFK Purification

procedure	vol (mL)	act. (units/mL)	protein (mg/mL)	sp act. (units/mg)	total units	yield (%)	purifn (x-fold)
100000*g* supernate from 600 g of liver	1960	0.63	31.0	0.020	1233	100	1
heat treatment	1640	0.55	9.2	0.060	900	73.0	3
$(NH_4)_2SO_4$ precipn	26.6	29.0	11.9	2.44	770	62.4	122
DEAE	102	5.8	0.62	9.35	592	48.0	468
Sepharose 2B	2.0	170	2.0	85	340	27.6	4250

centrifuged for 20 min at 17000*g*, and the supernatant solution is discarded. The pellet is resuspended in a minimum volume of column buffer (20 mM KP_i, 3 mM $MgSO_4$, 5 mM DTT, 20 μM EDTA, and 1 mM F6P, pH 7.6). The suspension, which is not entirely soluble, is dialyzed overnight against this buffer and centrifuged at 2000*g* for 15 min, and the pellet is discarded.

(*4*) *DEAE Chromatography*. Whatman DE-32 resin is equilibrated with the column buffer described above. A column is poured such that the bed volume measures at least 5 mL for every 100 g of liver used. The enzyme sample, diluted to a protein concentration of less than 5 mg/mL, is applied to the column. The column is then washed with the column buffer until no more protein is eluted. The PFK is eluted from the column with a linear gradient made from equal volumes of the column buffer and a buffer containing 300 mM KP_i, 3 mM $MgSO_4$, 5 mM DTT, 20 μM EDTA, and 1 mM F6P, pH 7.6. The total volume of the gradient should measure approximately 10 times the bed volume of the column.

The fractions containing significant PFK activity are pooled and concentrated by precipitating the protein with ammonium sulfate (final concentration was 55% of saturation). The pellet obtained by centrifuging at 17000*g* for 30 min is resuspended in 3–4 mL of column buffer. This suspension is spun at 2000*g* for 15 min to clarify.

(*5*) *Sepharose 2B Chromatography*. The solution is layered onto a Sepharose 2B column measuring 36 × 4 cm and eluted with column buffer. Three-milliliter fractions are collected. The PFK activity elutes soon after the void volume and a tail often extends into the lower M_r fractions. For maximum purity, a conservative cut is made by pooling only the fractions within the Gaussian portion of the peak. These pooled fractions are quickly concentrated to a volume of ~8 mL with an Amicon Diaflo ultrafiltration apparatus using an XM 50 membrane. The enzyme is then dialyzed overnight against storage buffer containing 20 mM KP_i, 3 mM $MgSO_4$, 1 mM F6P, 5 mM DTT, 100 μM EDTA, and 20% (w/v) glycerol, pH 7.6. The osmotic pressure difference between the sample and the buffer effects an additional concentration to ~5 mL during this dialysis. If desired, a further concentration can be achieved by burying the dialysis bag in dry Sephadex G-200. In general, increased concentration of the purified enzyme enhances its stability during storage. Purified rat liver PFK is quite stable when stored at 4 °C at a concentration of ~2 mg/mL in the glycerol-containing buffer described. It maintains full activity for at least 2 months.

Results

Data from a typical purification are shown in Table I. A greater than 4000-fold purification is achieved compared to the cytosolic extract, with a yield of 28%. The final specific activity of 85 units/mg measured at 25 °C compares favorably to the 87 units/mg measured at 30 °C obtained by Brand & Söling (1974) and the 95 units/mg measured at 37 °C obtained by Dunaway & Weber (1974). The purity of the resulting protein can be assessed by referring to the NaDodSO$_4$–polyacrylamide gel electrophoresis results shown in Figure 1.

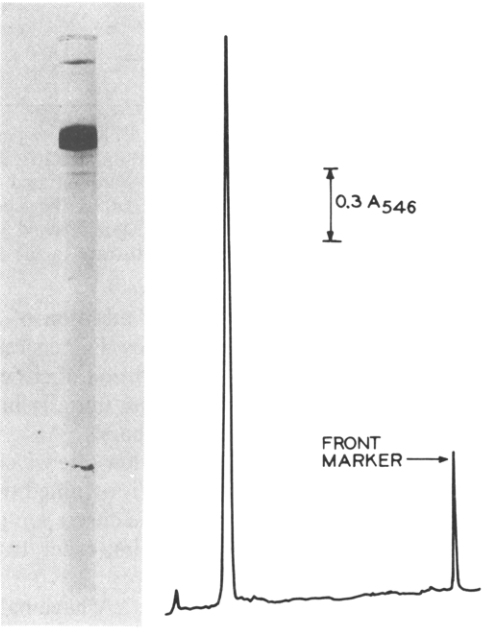

FIGURE 1: Results of NaDodSO$_4$–polyacrylamide disc gel electrophoresis performed on rat liver PFK purified as described in the text. The gel photographed contains ~25 μg of total protein stained with coomassie blue R. Direction of migration was from top to bottom with the dye front indicated by the ink marker. Absorption tracing was performed on a gel run identically except that for scaling purposes only half the amount of protein was applied to the gel.

1. Only slight traces of contamination are evident in the gel loaded with 25 μg of protein. A densitometry scan indicates that the major contaminating band represents about 1% of the total protein. When the migration of PFK was compared to that of known molecular weight proteins according to the procedure of Weber & Osborn (1969), the subunit molecular weight of PFK was determined to be approximately 82 000 ± 2000, in agreement with the results of Brand & Söling (1974) but significantly higher than the value of 65 000 reported by Dunaway & Weber (1974) using the same procedure.

We have performed this purification several times with similar results. The final specific activity usually falls within the range of 75–85 units/mg, depending somewhat upon the cut chosen after the Sepharose 2B column. The entire purification can be accomplished within 3 days, with the most cumbersome step being the initial 100000*g* spin of the homogenate if large amounts of starting material are used. The enzyme is relatively stable during the intermediate steps until it is eluted from the Sepharose column. At that point it is advisable to pool fractions, concentrate, and dialyze against the glycerol buffer as rapidly as possible. Some loss of activity is inevitable; however, this appears to be largely reversible upon concentration and dialysis as long as the time elapsed is kept to a minimum.

Assays of purified rat liver PFK demonstrate complex hysteretic behavior when performed under these conditions. Assays are generally nonlinear with accelerating or deceler-

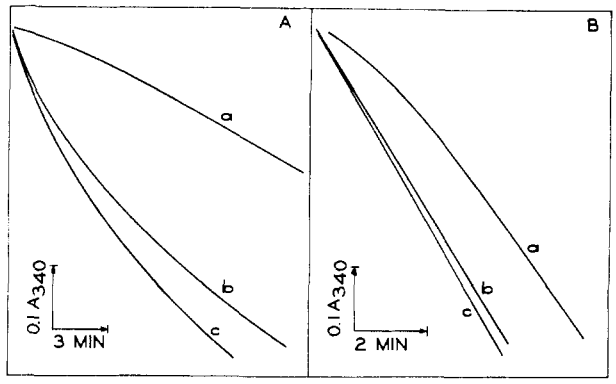

FIGURE 2: Artist's tracings of typical rat liver PFK assays performed at pH 7 with [MgATP] = 3 mM. For assays in panel A, [F6P] = 5 mM. In panel B, [F6P] = 12 mM. Assays were initiated with the addition of either F6P (a), ATP (b), or PFK (c). Other conditions of assay were identical and as described under Materials and Methods. PFK was added 2 min prior to the final substrate in (a) and (b).

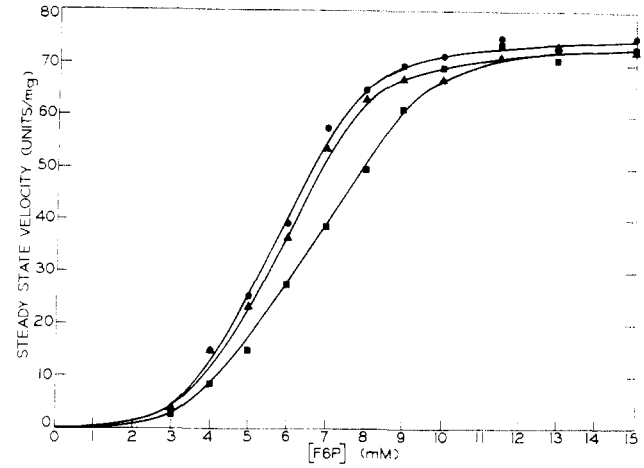

FIGURE 3: Steady-state activity of liver PFK as a function of [F6P]. Steady-state rates were determined from the curved assays by the nonlinear regression analysis described by Reinhart (1980). Assays were initiated by F6P (■), ATP (▲), or PFK (●).

ating rates depending on the order of addition of various components to the assay (Figure 2). At low F6P concentration (Figure 2A), assays initiated by the addition of either ATP, PFK, or Mg^{2+} (data not shown) show an initially high rate of reaction which decelerates with time (burst). Assays begun with the addition of F6P show an initially low rate which slowly increases (lag). For routine analysis, it is assumed the transients follow first-order behavior, and the curves are fitted to the general expression pertaining to first-order transients (Reinhart, 1980).

Both burst transients shown in Figure 2A have half-times of 2.3 min whereas the lag transient half-life is ~4.4 min. The ratio of the initial rate to the steady-state rate in the burst assays is about 4 whereas in the lag assay it is about 0.5. At high F6P concentration (Figure 2B), the assays initiated with either ATP or PFK show little if any curvature whereas the assay initiated with F6P shows an approximate 3.5-fold increase in reaction rate to that of the steady state with a transient half-time of 2.5 min.

In general, burst assays show a greater difference between initial and steady-state velocities and a decreasing transient half-life with decreasing F6P concentration. Lag assays have the opposite trend of increasing transient half-life and decreasing difference between initial and steady-state reaction rates with decreasing F6P concentration.

The lags referred to here are not the same as the lags observed in the assay of muscle PFK which have been attributed to the activation resulting from the build up of a steady-state concentration of FBP (Emerk & Frieden, 1975). Very high amounts of aldolase are used intentionally in our experiments to maintain a very low steady-state concentration of FBP (<1 μM). The only difference in the assays shown is the order of addition of the assay components. If the lag in Figure 2B, curve a, were due to FBP build up, a lag of similar magnitude should also be apparent in the otherwise linear assays b and c. By the same reasoning, the bursts in Figure 2A are not due to substrate depletion. The curvatures shown in Figure 2 are not due to some artifact of the coupling enzymes since similar results are obtained by using the pyruvate kinase-lactate dehydrogenase coupling system.

An additional conclusion to be drawn from Figure 2 is that the initial rates of these assays are highly dependent on the order of addition and hence do not reflect the steady-state activity of the enzyme produced by the conditions in the assay cuvette, but rather they seem to reflect the status of the enzyme prior to assay. The rate that is being approached with time is much more indicative of the steady-state activity brought about by the assay conditions which are the same in all cases in either part A or B of Figure 2. Consequently, the conventional approach of measuring initial velocities is not an appropriate method for evaluating these assays; rather, an evaluation which measures the rate after the transient is more correct. However, one must use a low amount of enzyme in assays of this type so that the total amount of substrate used up over the course of assay is insignificant.

The variation in the extrapolated steady-state rates obtained by using these three assay initiation procedures is shown in Figure 3. At both maximal activity (high F6P) and very low activity (low F6P) regions of the velocity profile, the steady-state velocities determined by the first-order fitting show reasonably good agreement regardless of the order of addition of the components to the assay. This is as expected since the steady-state rates should be independent of these factors. However, the agreement is not as good in the intermediate regions of F6P concentrations where the steady-state velocities of the assays that contain bursts are significantly greater than the corresponding rates following lags. This creates a shift in the F6P concentration required for half-maximal velocity from 6 mM in the case of the bursts to 7 mM in the case of the lags. This result could arise from a small degree of irreversibility associated with the preassay alteration of the enzyme to either an active or inhibited configuration, which apparently affects the affinity of the enzyme for F6P. A possible explanation for this observation will be considered in the third paper of this series (Reinhart & Lardy, 1980b).

The discrepancy in K_m[2] amounts to only ~15% and does not significantly influence the overall picture of the steady-state behavior of rat liver PFK when assayed under these conditions. The major characteristic of the data shown in Figure 3, which is apparent regardless of the assay procedure chosen, is that of an extremely high degree of cooperativity and high K_m with respect to F6P. The Hill coefficient under these assay conditions exceeds 4, and the K_m is ~6–7 mM. The combination of high positive cooperativity and high K_m results in the enzyme having very little activity at an F6P concentration of less than 0.1 mM, as occurs physiologically.

[2] "K_m" as used here represents merely the substrate concentration at which the enzyme exhibits half-maximal velocity. Additional meanings of K_m consistent with Michaelis–Menten formalism are not appropriate in this context due to the high degree of cooperativity.

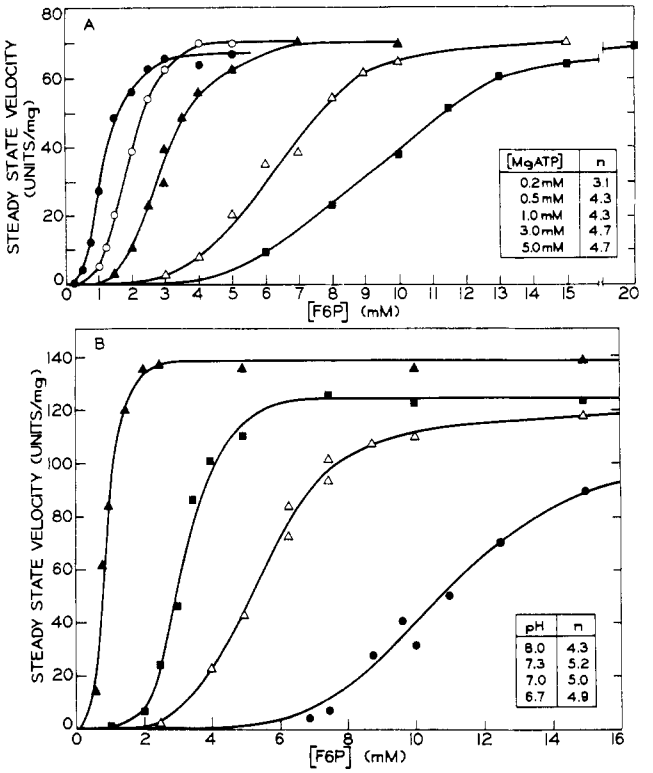

FIGURE 4: Influence of MgATP and pH on the steady-state activity of rat liver PFK. (A) MgATP concentrations: (●) 0.2; (○) 0.5; (▲) 1.0; (△) 3.0; (■) 5.0 mM. pH 7.0 at 25 °C. (B) [MgATP] = 3 mM. pH: (▲) 8.0; (■) 7.3; (△) 7.0; (●) 6.7. Temperature = 37 °C; similar results are obtained at 25 °C. Insets list Hill coefficients calculated from the data.

In an attempt to reconcile this observation with the required physiological activity of rat liver PFK, we examined the influence of various allosteric effectors of probable physiological significance. The effect of MgATP concentration on the steady-state activity of rat liver PFK as a function of F6P concentration is shown in Figure 4A. (For this and the experiments to follow, the assays were started by the addition of either ATP or PFK, resulting in burst assays.) As observed for the muscle isozyme, high MgATP concentration induces a positive cooperativity and an increased K_m in the F6P–velocity profile with relatively little effect on V_{max} once saturation of the catalytic binding site has been achieved. Unlike the muscle enzyme, however, the degree of cooperativity, as measured by the Hill coefficient, is extremely high, exceeding 4 at concentrations of MgATP greater than 0.5 mM. Even at 0.2 mM MgATP, where the nucleotide is approaching its catalytic K_m of 0.11 mM (Brand & Söling, 1974), the Hill coefficient is greater than 3. The K_m for F6P is greater than 1 mM whereas the true K_m for F6P, in the absence of ATP inhibition, is 0.08 mM (Brand & Söling, 1974). The binding of MgATP, presumably at an allosteric site, is thus extremely antagonistic to the binding of F6P. Since the hepatic concentration of MgATP in vivo is generally thought to lie in the range of 2–4 mM (Bergmeyer, 1974) and is also thought not to change substantially in various metabolic conditions, a fixed concentration of 3 mM MgATP was used in the kinetic experiments discussed below.

The dramatic effect of pH on the steady-state velocity–F6P profile is shown in Figure 4B. The affinity of the enzyme for F6P decreases when pH is decreased, but the cooperativity is not greatly affected. The Hill coefficient at pH 8 is greater than 4 and increases only slightly as pH is decreased. In contrast, the muscle enzyme is considered to perform essentially hyperbolically at pH 8 at MgATP levels less than 6 mM (Bloxham & Lardy, 1973). All following kinetic studies have been conducted at the physiological pH of 7.0. Millimolar citrate, the other important physiological inhibitor of liver PFK, further inhibits in the presence of 3 mM MgATP at pH 7 but has little effect below 0.2 mM (Figure 5). The effect is primarily an increase in K_m for F6P; however, 5 mM citrate does apparently inhibit V_{max} by ~20%. Cooperativity is not greatly affected, with a Hill coefficient close to 5 evident from 0.2 to 5 mM citrate.

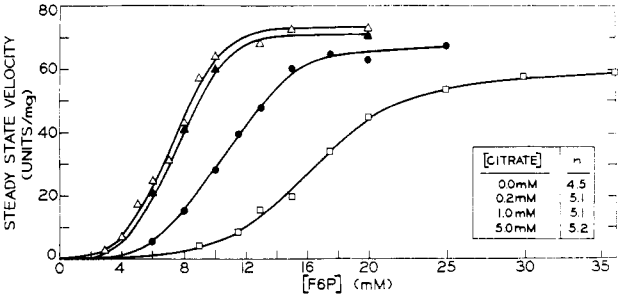

FIGURE 5: Inhibition by citrate of the steady-state activity of rat liver PFK at pH 7 and 3 mM MgATP. Citrate concentrations: (△) 0; (▲) 0.2; (●) 1.0; (□) 5.0 mM. Corresponding Hill coefficients are shown in the inset.

Of all the various positive effectors of PFK, probably the three most relevant to the enzyme's behavior in the liver cell are AMP, P_i, and FBP. The effect of each on liver PFK in the presence of physiological MgATP concentration and pH is shown in Figure 6. AMP causes a significant decrease in the K_m for F6P but has very little effect on V_{max} (Figure 6A). Even at saturating AMP (5 mM), however, the F6P K_m is greater than 2 mM and the Hill coefficient is greater than 3. Similarly, P_i (Figure 6B) slightly decreases the positive cooperativity while decreasing the K_m for F6P. But even at 10 mM P_i, the Hill coefficient is greater than 3 and the K_m is 1.2 mM. Once again V_{max} is only slightly increased. Only FBP shows a tendency to decrease the Hill coefficient significantly, to 1.6 at 260 μM FBP (Figure 6C). V_{max} is stimulated by ~10% and the K_m is lowered below 1 mM. The sensitivity of liver PFK to FBP is also ~1 order of magnitude greater than that for AMP or P_i.

The data presented in Figures 4–6 indicate that the essential characteristic of the action of the various effector ligands on rat liver PFK is the influence they exert on the binding of kinetically competent F6P. This response is similar to the actions of effectors on muscle PFK (Ramaiah, 1974). What is different is the high degree of positive cooperativity and high K_m for F6P still present at high levels of the positive effectors or low levels of negative effectors. This most likely reflects the increased sensitivity of the liver isozyme to inhibition by ATP as noted by others (Tsai & Kemp, 1974; Kemp, 1971).

The result of this high cooperativity and high K_m in the presence of 3 mM MgATP is that very little activity is demonstrated by PFK at F6P concentrations in the range (10–100 μM) found in the liver cell (see Discussion). To determine if cellular activity can be reconciled by combining the effectors studied here, we added each of the activators in various combinations at levels which represent the highest level ever likely to be encountered in the cell (Bergmeyer, 1974). In turn, the lowest reasonable level of citrate (0.2 mM) as well as the average value for MgATP concentration (3 mM) was present in all assays. The results (Figure 7) indicate that the activation induced is synergistic in that in the presence of any two effectors the K_m is lower than that which each individually promotes at saturation. Moreover, in the presence of all three

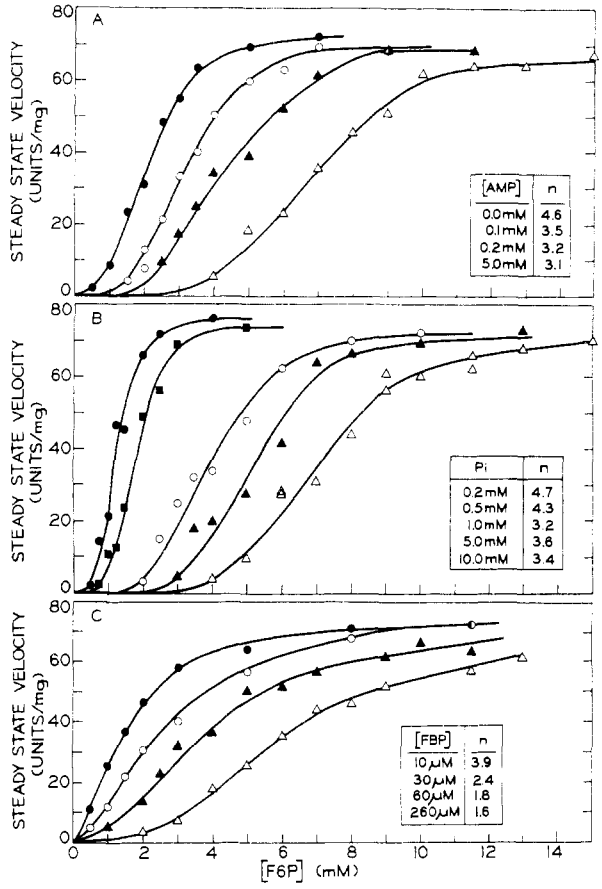

FIGURE 6: Influence of activators on the steady-state velocity–F6P profile for liver PFK at pH 7.0 and 3 mM MgATP. (A) AMP concentrations: (△) 0; (▲) 0.1; (○) 0.2; (●) 5.0 mM. (B) Activation by P_i; control assays without added P_i contain ~0.2 mM P_i from the PFK. Total P_i concentrations: (△) 0.2; (▲) 0.5; (○) 1.0; (■) 5.0; (●) 10 mM. (C) Activation by FBP. Pyruvate kinase–lactate dehydrogenase coupled assay was used, and the extent of reaction was limited to the production of 10 μM FBP during the assay. FBP concentrations at the end of the assay: (△) 10; (▲) 30; (○) 60; (●) 260 μM. Hill coefficients are listed in the insets.

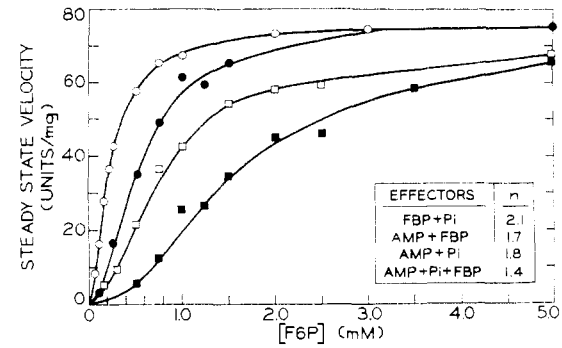

FIGURE 7: Synergistic activation of rat liver PFK by the simultaneous action of activators at physiologically optimum levels. The pyruvate kinase–lactate dehydrogenase coupling system was used, and the extent of reaction was limited to the production of an additional 10 μM FBP to the ligands initially present. *Initial ligand concentrations:* 30 μM FBP, 0.5 mM AMP, and 5 mM P_i. In addition, all assays contained 3 mM MgATP and 0.2 mM citrate at pH 7. Initial activator combinations: FBP plus P_i (■), AMP plus FBP (□), AMP plus P_i (●), AMP plus P_i plus FBP (○). Hill coefficients are listed in the inset.

activators, the enzyme experiences its lowest degree of cooperativity (1.4) and lowest K_m for F6P (0.24 mM). The synergistic effects of the three activators FBP, AMP, and P_i suggest that different binding sites exist for each ligand, as is the case for the muscle enzyme (Lowry & Passonneau, 1966).

Discussion

Ramaiah & Tejwani (1973) observed kinetic transients similar to those described here in their work with rabbit liver PFK and interpreted their results in terms of the allosterism model of Monod et al. (1965) where the slow interconversion of two enzyme conformations produced transients. We prefer to interpret these effects as reflecting the binding antagonism between F6P and MgATP, presumably at the active site and allosteric site, respectively. The enzyme in its steady-state configuration is inhibited at low F6P and not inhibited at high F6P. In the liver isozyme this inhibition is evidently a slower process than in the muscle enzyme and thus is seen during assay. What the rate-limiting step is in this inhibition mechanism, i.e., whether it is MgATP binding, F6P release, protein conformational change, etc., awaits further elucidation. It is evident, however, that the slowness of the hysteresis exhibited during assay is dependent upon both F6P and MgATP being present, since the effects on the initial rates, which are induced by either ligand alone, are very rapid. If the rate-limiting step were a conformational change of the protein in the absence of ligands, as proposed by Ramaiah & Tejwani (1973), then one would expect the inhibition of the initial rate, which results from placing "active" stock enzyme in excess of MgATP prior to initiating with F6P, to take as long to occur as the inhibition observed when the reaction is started with ATP. This is not the case, however, as reactions begun with F6P after a very short time of enzyme incubation with MgATP (<1 min) show complete inhibition of the initial rate, whereas burst transients generally have half-times greater than 2 min (Figure 2A). Indeed, Ramaiah and Tejwani's own data suggest a dependence of the rate of interconversion on the F6P concentration.

It should also be noted here that the inhibition of the initial rate of reaction is induced by MgATP and not free ATP. Stock enzyme stored in homogenization buffer (containing ATP but no Mg^{2+}) produces bursts when assayed at pH 7 and low F6P concentration. However, addition of Mg^{2+} to the stock causes assays to produce lags (data not shown). Also, as mentioned earlier, assays begun with Mg^{2+}, at low F6P concentration, result in bursts. These observations strongly suggest that MgATP has a much stronger inhibitory action than does free ATP for the liver isozyme. This is in contrast to the recently reported 10-fold higher affinity of the muscle enzyme for free ATP compared to MgATP (Pettigrew & Frieden, 1979).

In any case, the steady-state rates measured here appear to be relatively independent of the initial configuration of the enzyme and appear to represent the activity induced by the conditions of assay. The discrepancies which do exist at intermediate F6P concentrations represent minor deviations from the general picture of rat liver PFK as being highly cooperative and having low affinity for F6P under these conditions of pH and MgATP concentration.

As pointed out by Scrutton & Utter (1968), rat liver PFK is present in lower amounts than any other glycolytic enzyme and yet the rate of hepatic glycolysis is usually significantly lower than even this activity. The essential question is what is the normal rate of PFK turnover in the cell and can this rate be explained with the kinetic characteristics of the isolated enzyme described here?

The liver is capable of carrying out glycolysis in vivo under conditions of ample blood sugar supply, such as immediately after a large meal. Rates of glycolysis give a lower limit to the functional activity of PFK in the cell. However, actual

PFK turnover in the cell most likely exceeds the rate of glycolysis since significant "substrate cycling" is thought to occur between the PFK and FBPase reactions in the liver (Clark et al., 1974). Also, the measurement of lactate and pyruvate production generally underestimates the rate of glycolysis because of their continued metabolism.

Unfortunately, not a great deal of investigation has been conducted on hepatic glycolysis in vivo, largely because attention has been focused on the more specialized and important process of hepatic gluconeogenesis. Recently, Soboll et al. (1978) have measured rates of lactate and pyruvate production in perfused livers from both fed and 24-h fasted rats. Fed livers produced lactate and pyruvate from endogenous glycogen at a rate of 0.86 μmol of hexose equivalent per min per g of liver. Fasted livers exhibited very little endogenous glycolysis, due to glycogen depletion; however, glycolysis from 25 mM extracellular glucose achieved a rate of 0.55 μmol of hexose per min per g. Since maximal activity of the PFK present in the liver amounts to ~3.3 μmol/(min g) at 37 °C (Scrutton & Utter, 1968), the rates indicated above represent 26% (fed) and 15% (fasted) of the maximum PFK activity available, respectively.

In their investigation of FBPase–PFK cycling, Clark et al. (1974) attempted to measure directly the turnover of PFK, via the release of tritium from [5′-^{3}H]glucose, in hepatocytes isolated from 24-h fasted rats. Their results indicate that PFK turnover increases from 0.13 (4%) to 0.84 μmol/(min g) (25%) as extracellular glucose is raised from 5.5 to 27.4 mM. Even under highly glucoenogenic conditions, such as with dihydroxyacetone as the substrate, Clark et al. (1974) found PFK turnover of 0.16 μmol/(min g) or 5% of the available PFK activity.

It appears, therefore, that in isolated perfused liver or liver cells, PFK turnover varies in a range from a minimum of 4% to a maximum of at least 25% of the total PFK activity available. This activity is achieved with F6P levels ranging from 0.01 μmol/g in the cells of Clark et al. (1974) to as high as 0.1 μmol/g found in fed livers (Bergmeyer, 1974). Clearly, the affinity of PFK for F6P in the cell is significantly greater than that permitted by the average cellular concentration of MgATP (3 mM) on the assayable enzyme activity as indicated in Figure 4A.

As mentioned earlier, the action of each of the effectors FBP, AMP, and P_i is to increase the affinity of the enzyme for F6P in the presence of 3 mM MgATP. However, even near-saturating concentrations of each of these effectors, with the possible exception of FBP, fail to activate the enzyme sufficiently to permit it to function at physiological F6P levels. The curves defined by the data in Figure 6 indicate that, at 100 μM F6P, the activity of liver PFK is 4, 0.01, and 0.02% of V_{max} for 260 μM FBP, 5 mM AMP, and 10 mM P_i, respectively. It is only when the activators synergistically combine their efforts that the affinity for F6P begins to approach a level sufficient to account for physiological activity. For the maximum physiological levels of the activators shown in Figure 7, the activity of 100 μM F6P corresponds to 0.3% (FBP plus P_i), 3% (AMP plus FBP), and 5% (AMP plus P_i) of V_{max}. The action of all three activators stimulates activity to 23% of the maximum at 100 μM F6P.

The clear implication from these data is that ATP inhibition, which is generally more severe for the liver isozyme than the muscle isozyme (Tsai & Kemp, 1974), must be greatly alleviated in the cell. To a large extent this alleviation can be brought about by the combined action of the effectors examined here as shown in Figure 7. However, when the liver is called upon to produce its maximum glycolytic flux, it is unlikely that conditions in the cell, with respect to these effectors, will be as favorable as they are in the experiments of Figure 7. For example, maximum hepatic glycolytic flux is experienced in the fed state when glycolysis provides precursors for fatty acid synthesis (Newsholme & Start, 1973). However, the concentrations of ATP and citrate increase and that of AMP decreases with feeding (Soboll et al., 1978; Start & Newsholme, 1968). Consequently, the concentrations of these metabolites are not optimal for best activity when the enzyme needs to be most active. An additional mechanism to increase the enzyme's affinity for F6P, and hence its activity, is described in the following papers (Reinhart & Lardy, 1980a,b).

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Rat Liver Phosphofructokinase: Use of Fluorescence Polarization to Study Aggregation at Low Protein Concentration†

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ABSTRACT: The aggregation properties of rat liver phosphofructokinase (PFK) have been studied at low protein concentration by measuring the depolarization of fluorescence of PFK covalently labeled with pyrenebutyric acid. When PFK is labeled at a stoichiometry of between 0.3 and 1 pyrene per 3.2×10^5 daltons, kinetic integrity of the enzyme is maintained as determined by maximal specific activity and the cooperative response to fructose 6-phosphate (F6P) concentration at high MgATP concentration at pH 7. The fluorescence characteristics of the labeled enzyme (PB–PFK) are appropriate for the interpretation of polarization changes as reflecting changes in the average size, or rotational relaxation time, of the protein population. Consistent with the results of gel filtration and active enzyme ultracentrifugation experiments, the polarization data indicate that liver PFK is quite capable of forming very high molecular weight aggregates (50 S) at a protein concentration of 10 μg/mL. In isotonic pH 7 buffer containing excess Mg^{2+}, the dissociation constant of the high M_r aggregates is lower in the presence of saturating F6P than it is in the presence of saturating MgATP. In the absence of either substrate, PB–PFK dissociates past the tetrameric form of the enzyme with a concomitant loss in enzymatic activity. Subsequent addition of MgATP promotes tetramer formation much more than does the addition of F6P. These data are interpreted as indicating that the monomer–dimer population has a lower affinity for F6P than for MgATP whereas the high M_r aggregate population has a higher affinity for F6P than for MgATP.

In the preceding paper (Reinhart & Lardy, 1980a), we examined the kinetic properties of rat liver phosphofructokinase under conditions resembling those of the cell with respect to pH, ionic strength, and substrate and effector concentration. That study was an attempt to reconcile the kinetic properties of rat liver PFK[1] in vitro to the apparent behavior of the enzyme in vivo. The allosteric regulation of the liver enzyme under these conditions was found to influence the F6P binding to the enzyme. The major relevant antagonist to F6P binding is MgATP, and the other effectors act by either augmenting or diminishing this action. The actual cellular activity of PFK and its normal metabolic responses could be explained only by a greatly reduced influence of the MgATP inhibition under normal circumstances. This situation in turn could be approached only with the combined actions of several of the most important activators, each at optimum physiological concentrations.

One nonphysiological characteristic of those assays was the PFK concentration. Practical constraints on the performance of kinetic assays require the PFK concentration to be normally held from 0.04 to 0.1 μg/mL. In the cell, PFK is present at from 2 to 5 units/g of liver (at 37 °C), depending on age, nutritional state, etc. If we assume a specific activity of 80 units/mg (at 25 °C) and a water volume of 0.6 mL/g of liver (Tischler et al., 1977), this translates to a cellular PFK concentration in the range of from 20 to 50 μg/mL—2–3 orders of magnitude higher than the PFK concentration in the kinetic assays.

It has been known since the first isolation of rabbit muscle PFK (Ling et al., 1965) that the enzyme is capable of aggregating beyond the active tetramer (M_r 320 000) at high protein concentration. However, several groups of investigators have concluded that this aggregation does not occur significantly until the protein concentration exceeds 0.5–1 mg/mL (Pavelich & Hammes, 1973; Aaronson & Frieden, 1972; Leonard & Walker, 1972). The liver isozymes from various sources have also been found to aggregate, and the aggregation appears to be even more facile for the liver isozyme (Kemp, 1971). Trujillo & Deal (1977) have observed the formation of aggregates of pig liver PFK as large as 100 S, and the ability of rat liver PFK to aggregate is exploited in the Sepharose gel filtration step of the purification procedures of Brand & Söling (1974) and Reinhart & Lardy (1980a). However, these studies have largely been performed at enzyme concentrations in excess of 1 mg/mL—some 2 orders of magnitude greater than the cellular PFK concentration in the liver. In this and the following paper, we examine the aggregation behavior of rat liver PFK at low protein concentration in an attempt to bridge the gap between the kinetic experiments performed at a much lower than physiological enzyme concentration and the physical studies previously performed at a much higher than cellular enzyme concentration. To study protein aggregation in this concentration range systematically, it has been necessary to employ techniques that are much more sensitive than the more commonly used physical methods such as the Schlieren or Raleigh optics of a Model E ultracentrifuge. We

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[1] Abbreviations used: PFK, phosphofructokinase; F6P, fructose 6-phosphate; PBA, pyrenebutyric acid; PB–PFK, pyrenebutyrate–phosphofructokinase conjugates; DTT, dithiothreitol; P_i, inorganic phosphate; EDTA, disodium ethylenediaminetetraacetate; Mops, 4-morpholinepropanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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