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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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have found the technique of fluorescence polarization to be well suited to this application. Preliminary results utilizing this technique have been presented (Reinhart, 1978).

Materials and Methods

Materials were obtained from the following sources: ATP (sodium salt), P-L Biochemicals; F6P (sodium salt) and DTT, Sigma Chemical Co.; pyrenebutyric acid, Molecular Probes; Bio-Gel A-5m, Bio-Rad; rabbit muscle phosphofructokinase (used without further purification), Boehringer Mannhein. Glycerol used in fluorescence experiments was spectrograde obtained from Eastman. All other reagents and chemicals were of analytical grade and deionized, distilled water was used throughout the experiments.

Rat liver PFK was purified, and kinetic assays at pH 8 and pH 7 were performed as described in the previous paper (Reinhart & Lardy, 1980a). Homogenization buffer includes 50 mM Tris-HCl, 50 mM NaF, 5 mM DTT, and 1 mM ATP at pH 8.0. Storage buffer consists of 20 mM KP_i, 3 mM $MgSO_4$, 5 mM DTT, 1 mM F6P, 100 μM EDTA, and 20% (w/v) glycerol, pH 7.6. In addition, the polarization and gel filtration experiments described were performed in an isotonic buffer containing 50 mM Mops–KOH, 100 mM KCl, 5 mM $MgCl_2$, 100 μM EDTA, and 2 mM DTT at pH 7.0 plus the addition of either F6P or ATP as indicated. When the concentration of ATP was other than 3 mM, the total concentration of $MgCl_2$ was adjusted to a level 2 mM in excess of the total ATP concentration. Experiments "in the absence of ligands" were performed in the above buffer with no further additions.

Conjugates of rat liver PFK and pyrenebutyric acid were prepared as follows. Dimethylformamide–SO_3 was prepared according to the procedure of Warme & Hager (1970), and a mixed anhydride between PBA and sulfuric acid was prepared in dimethylformamide as described by Rawitch et al. (1969). An appropriate aliquot of this mixed anhydride solution was then allowed to react with purified PFK in its normal storage buffer. A typical procedure used to effect a labeling stoichiometry of approximately 1 pyrene per 3.2×10^5 daltons was as follows: 20 μL of mixed anhydride diluted 1:100 in PFK storage buffer was added to 0.5 mL of PFK (2 mg/mL), mixed, and allowed to react for 15–30 min at room temperature. The reaction was stopped by desalting the mixture using a small (5 mL) Sephadex G-50-80 column and the centrifuge column procedure of Orly and Selinger as described by Penefsky (1977). The labeled PFK solution was then dialyzed for $\sim$1 week against five 100-mL changes of storage buffer supplemented with 100 mM Tris-HCl (pH 7.6) at room temperature. Afterward the enzyme was once again desalted into storage buffer by using the centrifuge column procedure and refrigerated. Stoichiometry was determined assuming a pyrene extinction of 4×10^4 at 346 nm (Rawitch et al., 1969).

Fluorescence measurements were obtained on an SLM Instruments Model 8000 photon counting spectrofluorometer equipped with a dual-grating excitation monochromator and thermostated at 25 °C. Fluorescence polarization experiments were performed with Glan-Thompson prism polarizers installed in a t-format configuration. The instrument in this configuration is similar in both design and performance to the one described by Jameson et al. (1978). Emission was monitored through sandwiched 0-51 and 7-54 Corning glass filters placed between the sample and each phototube. Excitation was at 346 nm with slits set at 2-nm band-pass.

Polarization (P) was determined in the following manner. Fluorescence intensity was measured simultaneously through polarizing filters oriented parallel ($I_\parallel$) and perpendicular ($I_\perp$), respectively, to the vertical laboratory axis through the opposite faces of the cuvette, 90° to the excitation beam. Emission intensities were adjusted with neutral density filters (silver-coated glass from Corning) placed in the excitation beam so as to provide a counting rate of less than 100 KHz in all cases to avoid errors due to pulse pile up. Appropriate blanks were subtracted to correct for H_2O Raman scattering, dark current, and background fluorescence due to contaminants of buffer components. These measurements were combined in the following manner:

$$R = [I_\parallel - I_\parallel(\text{blank})]/[I_\perp - I_\perp(\text{blank})] \quad (1)$$

This ratio was first determined with exciting light vertically polarized (R_V) and then determined with the excitation polarizer positioned horizontally, parallel to the direction of emission observation (R_H). This latter measurement provides a normalization factor to correct for performance discrepancies in the two different emission optical arms since in theory $I_\parallel = I_\perp$ in this case (Weber & Bablouzian, 1966). Polarization is then determined by computing the quantity (Jameson et al., 1978)

$$P = (R_V/R_H - 1)/(R_V/R_H + 1) \quad (2)$$

The ratio of rotational relaxation time to fluorescent lifetime (ρ/τ) was calculated according to the Perrin equation as elaborated for solutions of macromolecules by Weber (1952a,b):

$$(1/P - 1/3) = (1/P_0 - 1/3)(1 + 3\tau/\rho) \quad (3)$$

The limiting polarization in the absence of macromolecular rotations (P_0) for the PB–PFK conjugates under various conditions was determined by extrapolating to infinite viscosity a "Perrin plot" of $1/P$ vs. T/η as described by Weber (1952a). Viscosity (η) was increased at constant temperature by adding glycerol. Literature values for viscosity were used (Dean, 1973) assuming a negligible influence of the salts and other components of the buffer.

Light-scattering experiments were performed with 90° geometry on the SLM spectrofluorometer with the emission monochromator in place. Both emission and excitation monochromators were set at 360 nm with 2-nm slits. Emission intensities were measured ratiometrically to correct for fluctuations in lamp intensity. All solutions except for the stock enzyme solution were filtered through a 0.45-μm Millipore filter to remove dust. Blank readings were taken in the same cuvette prior to addition of enzyme so that net scatter of the added protein could be determined.

Fluorescent lifetimes were measured at 25 °C on a cross correlation phase fluorometer described by Spencer & Weber (1969) modified with updated electronics from SLM Instruments. Lifetimes were calculated from the degree of demodulation of the fluorescence signal relative to that of a glycogen scattering suspension. The sample was excited with light at 346 nm and modulated at a frequency of 6 MHz, chosen because of the long lifetime being measured. Pyrene emission was measured through the same Corning 0-51 and 7-54 filter pair that was used in the polarization experiments.

Gel filtration experiments were performed with a 1.4×39 cm Bio-Gel A-5m (100–200 mesh) column equilibrated with the buffer indicated in the text. The column was run at 4 °C and 0.6-mL fractions were collected and assayed for PFK activity by using the standard pH 8 assay. Experiments at high protein concentration were performed by layering a 0.5-mL sample on top of the column and immediately beginning fraction collection. Experiments at low protein concentration were performed using the continuous elution

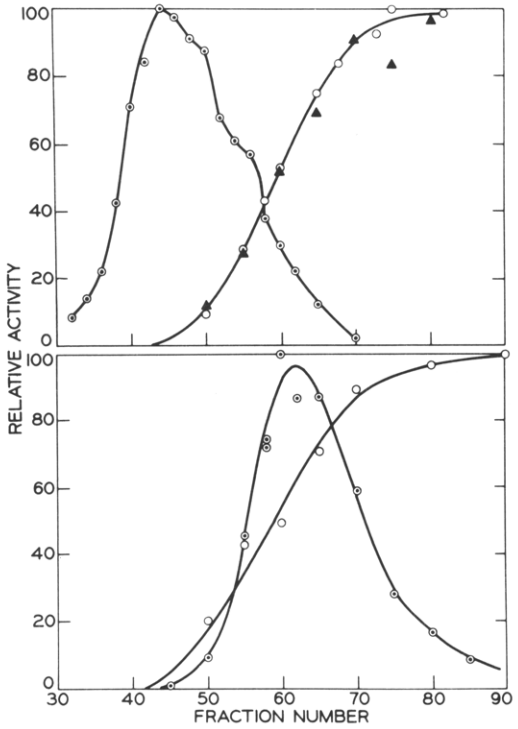

FIGURE 1: Comparison of gel filtration behavior of liver and muscle PFK. All experiments shown were run on the same Bio-Gel A-5m column. Top panel: rat liver PFK run in homogenization buffer at 50 μg/mL (⊙), isotonic pH 7 buffer with 3 mM MgATP at 0.1 μg/mL (○), or isotonic pH 7 buffer with 10 mM F6P at 0.1 μg/mL (▲). Bottom panel: rabbit muscle PFK dialyzed against and run in homogenization buffer at 250 μg/mL (⊙) or isotonic pH 7 buffer with 3 mM MgATP at 0.1 μg/mL (○).

technique of Pavelich & Hammes (1973) where the sample is continuously applied until the protein concentration, or in this case PFK activity, of the effluent is the same as that of the applied sample. In this procedure the midpoint of the eluting front is equivalent to the elution position of the conventional procedure.

Active enzyme analytical-band ultracentrifugation was performed essentially as described by Cohen & Mire (1971) in a Beckman Model E ultracentrifuge. Twenty microliters of PFK (10 μg/mL in isotonic pH 7 buffer including 1 mM F6P) was placed in the side well of a Beckman Vinograd cell. The main compartment of the cell was filled with all of the usual components necessary for pH 7 assay including 20 mM F6P, 3 mM MgATP, 0.5 mM NADH, and the coupling enzymes aldolase (100 μg/mL) and triosephosphate isomerase–glycerol-3-phosphate dehydrogenase (50 μg/mL). The sample was spun in a Beckman AN-D rotor at 28 000 rpm at 20 °C. The 365-nm line of a low-pressure Hg lamp was used to illuminate the cell. Density tracings of the resultant photographic negatives, made with a Joyce Loebl MK III C recording densitometer, were used to measure the sedimentation of the enzyme. No corrections of the sedimentation values were made for the presence of buffer constituents since the ionic strength was approximately 0.2 and corrections would be minor.

Protein concentrations were determined by using the method of Lowry et al. (1951). A gravimetrically prepared solution of bovine serum albumin, fraction V, was used as a standard.

Results

The high tendency of rat liver PFK to aggregate to polymers greater than tetramer can be seen in the gel filtration experiments described in Figure 1. A sample of partially purified liver PFK, initially containing ~50 μg/mL enzyme in homogenization buffer, elutes substantially ahead of the same enzyme sample diluted to 0.1 μg/mL in the isotonic pH 7 buffer, described under Materials and Methods, containing either MgATP or F6P. This latter enzyme concentration is similar to that normally used during kinetic assay. The elution position of the dilute enzyme corresponds to the expected position of the tetrameric enzyme since it occurs just behind that of ferritin which has a molecular weight of 500 000 (data not shown).

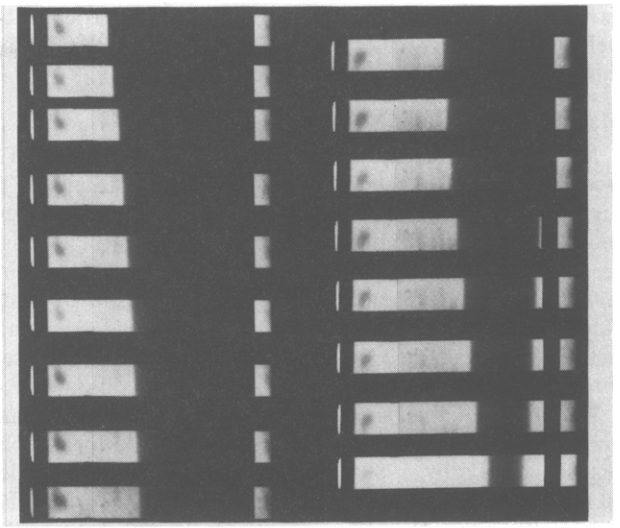

FIGURE 2: Active enzyme analytical-band ultracentrifugation of rat liver PFK performed as described under Materials and Methods. Sedimentation is from left to right with photographs chronologically ordered from picture 1 to 9 down the left and then from picture 10 to 17 down the right. The time between photographs is 4 min until picture 12 and 8 min thereafter. Lighter areas indicate solution rendered transparent at 365 nm due to the oxidation of NADH resulting from the presence of PFK. Note the two advancing boundaries evident in pictures 1–7 as well as the pileup of PFK activity at the bottom of the cell beginning in picture 12.

This aggregation behavior can be contrasted to that of the rabbit skeletal muscle isozyme (Figure 1, bottom). Partially purified muscle PFK at ~250 μg/mL in homogenization buffer elutes at the same position as does the enzyme diluted 2000-fold to 0.1 μg/mL in the isotonic pH 7 buffer containing 3 mM MgATP. Both enzyme concentrations elute as expected for the tetrameric enzyme, consistent with the findings of Pavelich & Hammes (1973), Aaronson & Frieden (1972), and Leonard & Walker (1972). These data directly demonstrate the greater tendency of the liver enzyme, relative to muscle PFK, to aggregate to species larger than the tetramer at enzyme concentrations less than 0.25 mg/mL.

The capability of rat liver PFK to aggregate to high M_r polymers even at low protein concentration is further demonstrated by the results shown in Figure 2. Using the procedure of Cohen & Mire (1971), the sedimentation of liver PFK at a concentration of 10 μg/mL was observed by following the activity of the enzyme rather than that of the protein itself. PFK was preincubated with F6P at pH 7 and assayed during sedimentation under maximal velocity conditions at pH 7. In the first seven photographs, two species appear to separate as the sedimentation progresses. The slower of the two continues to sediment consistently throughout the experiment at a rate of 14 S which is comparable to the sedimentation rate reported for the rabbit muscle PFK tetramer (Ling et al., 1965). Progress of the faster species appears to cease after picture 7. However, the continued existence of this species is revealed by the appearance of ki-

netically active material at the bottom of the cell beginning in picture 12. The rate of sedimentation for the heavy species, indicated by the first few photographs, is 50 S. A species of this size would be expected to reach the bottom of the cell at approximately picture 12.

It is unclear whether the undetectability of this heavy species in the middle photographs is due to diffusion to a concentration below the detection limit of the film or to inhibition of the enzymatic activity as the species progresses through the cell. Indeed, this illustrates a major problem of any physical method which relies upon kinetic activity for detection. Accurate quantitation of relative amounts of various protein species is dependent upon accurate knowledge of the specific activities of the various enzyme forms. Also, inactive forms, such as the monomer and dimer of PFK, are not detectable by these methods. These considerations led to the desire to establish a system for studying the aggregation behavior of rat liver PFK which was very sensitive yet independent of enzymatic activity. A technique with suitable sensitivity is that of fluorescence polarization.

Since the fluorescent lifetimes of endogenous tryptophan and tyrosine residues are much too short to be employed in polarization studies of this enzyme, it was necessary to attach the fluorescent probe pyrenebutyric acid (PBA) covalently to purified rat liver PFK. PBA is the longest lifetime fluorescent probe currently available and was first used for fluorescent polarization studies of proteins by Knopp & Weber (1967, 1969).

Covalent attachment of the pyrene moiety to purified rat liver PFK following the procedure of Rawitch et al. (1969) was confirmed by $NaDodSO_4$ gel electrophoresis (data not shown). When the extent of pyrene incorporation is between 0.2 and 1 pyrene per 3×10^5 daltons, the optimal specific activity of the enzyme is unchanged. The kinetic properties with respect to F6P concentration at pH 7 and high nucleotide concentration are very similar to those of the native enzyme (data not shown). Since the kinetic behavior of rat liver PFK under these conditions is quite sensitive to a wide variety of ligands (Reinhart & Lardy, 1980a), the relatively minor kinetic influence of the bound pyrene supports the idea that properties of the labeled enzyme are similar to those of the native enzyme.

Figure 3A illustrates the polarization behavior of PB–PFK fluorescence in response to the substrates MgATP and F6P at a protein concentration of 7 μg/mL. Under normal storage, the labeled enzyme is exposed to 1 mM F6P at high protein concentration (0.7 mg/mL). Upon dilution into the normal isotonic pH 7 buffer in the presence of 5 mM F6P, the polarization remains quite high (ca. 0.150) and very stable for 3 h. When the enzyme is diluted into an identical buffer except for the substitution of 3 mM ATP for the F6P (Mg^{2+} is always present in excess of the nucleotide), the polarization immediately decreases to ca. 0.105 and gradually declines to 0.095 in the next 3 h. If the enzyme is diluted in the absence of ligands (except for K^+, Mg^{2+}, and 10 μM F6P carried over with the enzyme), the polarization is lower yet and continues to decrease during the course of the first hour until a very low value of 0.042 is obtained. If F6P is added to this sample after 55 min, as indicated by the arrow, only a small increase of the polarization occurs over the next 2 h. However, if ATP is added after 55 min, the polarization increases a substantial amount.

The optimal assayable specific activity of labeled enzyme incubated in parallel fashion can be seen in Figure 3B. PB–PFK at 7 μg/mL maintains nearly full activity over the course

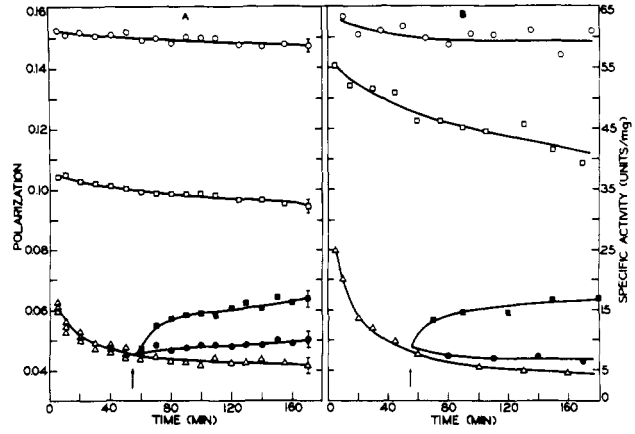

FIGURE 3: Polarization and specific activity of PB–PFK incubated at 7 μg/mL in the presence and absence of substrates. Each polarization value in panel A is the average of six measurements obtained over the course of 5 min beginning at the time indicated. The average standard deviation of these values is indicated by the error bars on the final point of each curve. Specific activities shown in panel B were measured at pH 8 under optimal conditions. At time 0, PB–PFK was diluted into isotonic pH 7 buffer containing 5 mM F6P (○), 3 mM MgATP (□), or no added substrate (△). 5 mM F6P (●) or 3 mM ATP (■) was added to the latter experiment 55 min after PB–PFK addition, indicated by the arrow.

of 3 h when diluted in the presence of 5 mM F6P. With 3 mM MgATP, the activity is initially 12% less than that in the F6P case and slowly, though significantly, is lost until after 3 h the activity of the MgATP incubation is 60% of the corresponding activity in F6P. In the absence of either ATP or F6P, however, the specific activity decreases rapidly to less than 20% of the activity in F6P within the first half hour and continues to decrease over the next 2.5 h. The time dependence of this decrease in activity is similar to the time course of the decrease in polarization seen in Figure 3A. This implies that the polarization decrease in the absence of ligands reflects an inhibition of the enzyme which is maintained during assay under optimal conditions. Subsequent addition of either 3 mM ATP or 5 mM F6P at 55 min (see arrow) results in a moderate rise in activity upon the addition of ATP but only a partial stabilization of the remaining activity upon the addition of F6P. These changes are also reflected in the corresponding polarization changes seen in Figure 3A.

Before these changes in polarization can be interpreted as reflecting changes in the size of the protein, several alternative explanations for this result should be examined. From the Perrin equation (eq 3), it can be seen that changes in fluorescent lifetime will cause changes in polarization. In addition, any conformational change which would result in a greater degree of locally available rotational freedom of the pyrene moiety would decrease the polarization of the pyrene fluorescence, independent of the rotation of the protein molecule to which it is attached (Weber, 1952a). These considerations are of particular importance to the interpretation of the polarization differences observed between the MgATP and F6P situations since these ligands are known to alter the conformation of muscle PFK (Bloxham, 1973; Pettigrew & Frieden, 1979).

Fluorescent lifetimes of the labeled enzyme in the presence of either F6P or MgATP were measured, and the results are given in Table I. If heterogeneity of the emitting population exists, these lifetimes may be longer than the weighted average lifetimes of the various emitting species (Spencer & Weber, 1969). The values obtained are longer than the lifetimes (100 ns) obtained for pyrenebutyric conjugates prepared by Knopp & Weber (1969); however, they are only slightly greater than

Table I: Fluorescent Lifetime of PB-PFK in the Presence of Either Substrate[a]

parameter	substrate	
	5 mM F6P	3 mM MgATP
rel intensity	5.29	5.32
polarization	0.1432 ± 0.0010	0.1146 ± 0.0015
lifetime	242 ± 2 ns	226 ± 5 ns

[a] Measurements were performed as described in the text on a solution containing 50 μg of PB-PFK per mL in isotonic pH 7 buffer containing the indicated substrate. Intensity, polarization, and lifetime measurements were all performed on the same samples, using the same emission filters.

the values (200 ns) obtained by Vaughan & Weber (1970). These values are therefore reasonable and probably indicative of a pyrene environment which stabilizes the excited state and protects it somewhat from quenching by small molecules such as O_2.

For comparative purposes the absolute value of the lifetime is not as important as the relative values in the presence of MgATP and F6P. The lifetimes in Table I differ by less than 7% which cannot account for the substantial polarization difference. These data, plus the observations that the emission spectral characteristics and quantum yield of the pyrene moiety on the labeled enzyme are not significantly different in the presence of MgATP compared to F6P (data not shown), lead to the conclusion that the polarization effects observed are not due to electronic or lifetime perturbations of the pyrene.

The presence of intramolecular rotations can be tested experimentally because they will be largely independent of solvent viscosity (Weber, 1952a,b). Consequently, the change in polarization will be reflected in a commensurate change in the limiting polarization, P_0, determined by the y intercept of a plot of $1/P$ vs. T/η where the latter quantity is determined by isothermally varying the viscosity of the medium with glycerol. These experiments were performed with PB–PFK in the presence of F6P, MgATP, and no other ligands, and the results can be seen in Figure 4. The P_0 values obtained are the same within experimental error, indicating that polarization changes are not due to a conformational change influencing the rotational environment of the pyrene. Moreover, the weighted average value of P_0 determined from the three lines, 0.164 ± 0.001, is close to the value of 0.178 ± 0.002 determined for PBA in propylene glycol at −55 °C. Similar experiments with other PB–PFK preparations have yielded P_0 values of 0.172 and 0.174, with the differences most likely attributable to varying trace amounts of free PBA. These results indicate that the pyrene is very rigidly bound to the protein under these conditions.

The slopes of the lines in Figure 4 are related to molecular volume by the Perrin equation. The relative molecular volumes of the protein for the three cases in Figure 4 are no ligand/MgATP/F6P equal to 1:4:20 assuming a constant fluorescent lifetime.

Further confirmation that the polarization changes reflect size changes of the protein molecule can be seen in the 90° light-scattering data of Figure 5. The greater light scattering observed in the presence of F6P compared to MgATP, and in turn MgATP greater than no added ligands, represents a molecular weight relationship similar to that reflected in the polarization data of Figures 3 and 4. The light-scattering data also confirm that the aggregation behavior of the PB–PFK conjugates is comparable to that of native PFK and do not just reveal the peculiar behavior of the small subset of the total protein population which is actually labeled.

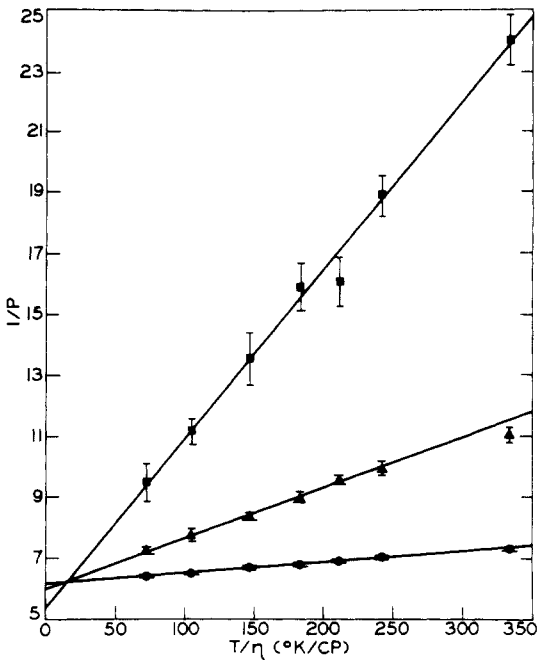

FIGURE 4: Perrin plot of PB–PFK in the presence or absence of substrate. The quantity τ/η was varied isothermally by the addition of glycerol. PB–PFK was incubated for 3 h at 7 μg/mL prior to the experiment. Values represent averages of at least 12 measurements with the standard deviation indicated. Isotonic pH 7 buffer contained either 5 mM F6P (●), 3 mM MgATP (▲), or no additional ligands (■).

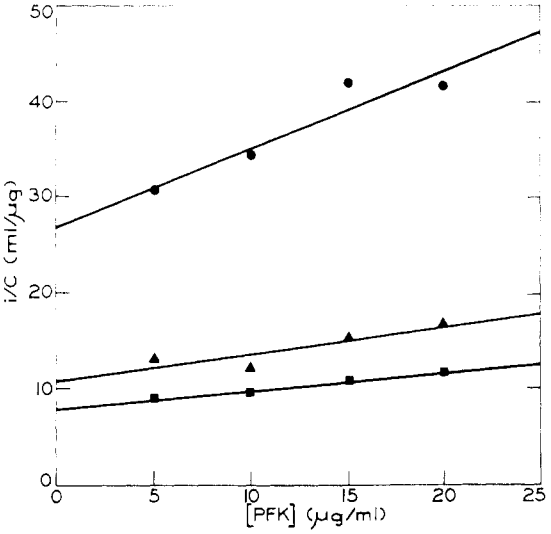

FIGURE 5: Light-scattering behavior at 360 nm of rat liver PFK in the presence or absence of substrate at 25 °C. Scattering intensity normalized to protein concentration is expressed in arbitrary units. Isotonic pH 7 buffer contained either 5 mM F6P (●), 3 mM MgATP (▲), or no additions (■).

It seems appropriate, therefore, to interpret the polarization of PB–PFK as reflecting actual changes in size, and hence aggregation state, of the protein. This relationship is quantitatively described by the Perrin equation (eq 3) where the rotational relaxation time is directly proportional to protein molar volume (see Discussion).

The effect of enzyme concentration on the rotational relaxation time of PB–PFK conjugates in the presence of either substrate can be seen in Figure 6. The dashed line corresponds to the predicted value of ρ/τ for the PFK tetramer (see Discussion). Unfortunately, due to the practical limitations of enzyme availability as well as the near P_0 polarizations

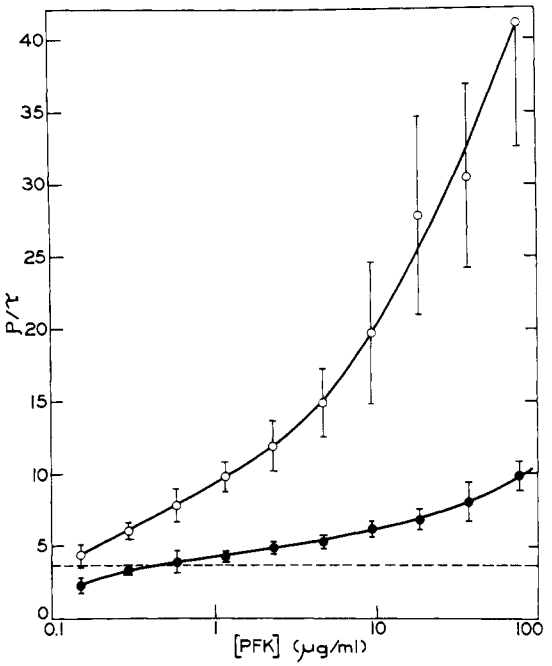

FIGURE 6: Dependence of the ratio of rotational correlation time to fluorescent lifetime (ρ/τ) on the concentration of PB–PFK in the presence of either 5 mM F6P (○) or 3 mM MgATP (●). The large standard deviations at large ρ/τ are a consequence of the close proximity of polarization values to P_0. The dashed line corresponds to the theoretical ρ/τ of the tetramer of rabbit muscle PFK based upon a Stokes' radius of 67 Å and a fluorescent lifetime of 230 ns (see Discussion).

obtained at high concentrations, complete dissociation curves could not be obtained. However, the data indicate a clear propensity of rat liver PFK to aggregate to average sizes larger than the tetramer at concentrations beginning at or below 1 μg/mL. Moreover, the average dissociation constant of these high M_r species is clearly lower in the presence of F6P than in the presence of MgATP.

Discussion

For a spherical molecule, rotational relaxation time, ρ, is related to the molar volume by the relationship

$$\rho = 3\eta V/(RT) \quad (4)$$

where η is solvent viscosity, V is molar volume, T is absolute temperature, and R is the gas constant (Weber, 1952a). With this equation, one can calculate the theoretical rotational relaxation time for a molecule of known Stokes' radius. With appropriate substitution of constants, eq 4 becomes

$$\rho = (2.73 \times 10^{-3})r^3 \quad (5)$$

where r is the Stokes' radius measured in angstroms and ρ is given in nanoseconds.

Pavelich & Hammes (1973) have determined the Stokes' radius of rabbit muscle PFK to be 67 Å. Since the molecular weight of the muscle isozyme monomer (M_r 80 000) is essentially the same as that of the liver enzyme monomer, one might expect the tetramer of the liver enzyme to be of a similar shape and size and hence have a similar Stokes' radius. This similarity is also indicated by the comparable gel filtration and sedimentation behavior of the tetramer of the two isozymes (Figures 1 and 2). By use of eq 5, the rotational relaxation time of a molecule with a Stokes' radius of 67 Å is 821 ns. If the lifetime of the pyrene is 230 ns (Table I), the value of ρ/τ is 3.6. This value corresponds to the dashed line in Figure 6.

The predicted rotational relaxation time for the muscle tetramer appears to very nearly correspond to the limiting rotational relaxation time being approached by dilution of the liver isozyme in the presence of either MgATP or F6P. The fact that the enzyme is approaching similar values in both cases is consistent with the gel filtration results shown in Figure 1 which indicate that the enzyme behaves as a tetramer at 0.1 μg/mL in the presence of either MgATP or F6P. The noticeable deflection to lower values at the lower concentrations of the MgATP curve (Figure 6) probably reflects a dissociation of the tetramer at these low protein concentrations. It is likely that the dissociation constant for the tetramer is beginning to make a significant contribution to the equilibrium mixture of protein sizes that exist below the microgram per milliliter concentration range.

It is also apparent from Figure 6 that liver PFK begins to aggregate significantly in the presence of MgATP at protein concentrations above 1 μg/mL. This is consistent with the appearance of a Schlieren peak of 50 S appearing at 1 mg/mL in the presence of either MgATP or F6P (data not shown). The differential effects of the substrates on the aggregation behavior are therefore masked at high protein concentration but readily apparent when studied at low protein concentration.

Several investigators have concluded that rabbit muscle PFK aggregates have the same specific activity as the tetramer, which is the smallest active form of the enzyme (Lad et al., 1973; Aaronson & Frieden, 1972; Pavelich & Hammes, 1973). They have also concluded that the dissociation of the aggregates is rapid whereas the dissociation of the tetramer is slow. For these reasons it has been concluded that kinetic assays reflect the activity of the enzyme as tetramers (Lad et al., 1973). The tetramers do not significantly dissociate, which would cause a commensurate loss in activity, during the short duration of the assay. If these characteristics are true for the liver enzyme as well, the data shown in Figure 3 can be interpreted as follows.

The labeled stock enzyme, usually about 0.7 mg/mL in protein concentration, is highly aggregated. When this is diluted 100-fold in the presence of 5 mM F6P (and the other buffer constituents at pH 7), much of the aggregation is maintained as evidenced by the high polarization. The activity at pH 8, which can be thought of as an assay for tetramer plus aggregates, shows that the enzyme exhibits nearly full activity in this state. Both the polarization and activity are very stable over a 3-h period. Dilution in the presence of saturating MgATP (3 mM) produces a much lower polarization with only a small decrease in activity initially. The initially lower polarization is consistent with the PFK population containing fewer aggregates and more tetramers at this protein concentration with MgATP (100% tetramers would produce a polarization of 0.095 in this system). Since the concentration of tetramers has increased, the concentration of dimers and monomers, though small, would be greater in the presence of MgATP than F6P. Hence, the activity of PFK in MgATP, initially close to that in F6P, slowly decreases due to the slow process of tetramer dissociation which is also indicated by the slow polarization decrease.

When PB–PFK is diluted at pH 7 in the absence of ligands, the enzyme quaternary structure is unstable, and the aggregates dissociate rapidly. The polarization falls to a very low level as does the activity, indicating a large degree of tetramer dissociation also. The predicted polarization of the dimer is 0.046 based on the 44-Å Stokes' radius determined by Pavelich & Hammes (1973). Subsequent addition of MgATP causes a significant increase in polarization and activity consistent

with a shift in the tetramer–dimer equilibrium toward tetramer. This influence of MgATP is similar to its known property of promoting and stabilizing tetramer formation in the muscle enzyme (Lad et al., 1973). Addition of F6P, on the other hand, has only a slight effect on both activity and polarization, indicating only a minor shift in the tetramer–dimer equilibrium.

F6P exhibits, therefore, the ironic behavior of stabilizing a high degree of aggregation while at the same time not promoting a significant amount of aggregation from a mixed monomer–dimer population. One possible explanation for this is as follows. With either F6P or MgATP, the liganded form of the enzyme dissociates less readily to protomers than does the unliganded form. This in turn implies that the dissociation constant of the ligand from the tetramer and aggregate forms of the enzyme is less than that of the dissociated forms (Weber, 1971, 1972, 1975). In the case of F6P, the affinity of the dissociated forms is sufficiently low that reaggregation upon subsequent addition of F6P is very slow. The affinity of the monomer–dimer mixture for MgATP is not so low, and subsequent addition of this ligand causes a more rapid increase in polarization due to the aggregation promoted by ligand binding.

Of the two liganded forms of the enzyme, the F6P form has the greater tendency to aggregate beyond the tetramer to form the higher order aggregates. These higher order aggregates consist of the 50S species seen in Figure 2 as well as probably intermediate forms between 14 S and 50 S. Mechanistic and physiological ramifications will be discussed more thoroughly in the following paper (Reinhart & Lardy, 1980b).

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