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## Sedimentation Study of a Catalytically Active Form of Rabbit Muscle Phosphofructokinase at pH 8.55<sup>†</sup>

Lyndal K. Hesterberg and James C. Lee\*

**ABSTRACT:** The enzymatic active form of rabbit muscle phosphofructokinase (PFK) was observed directly by using the method of reacting or active enzyme centrifugation (AEC). These studies were performed in two assay systems: a coupled enzyme and a pH-dependent dye-linked system in glycylglycine buffer at pH 8.55 and  $23 \pm 1$  °C. The sedimenting band of PFK was stabilized by three solvent systems: 50% (v/v) D<sub>2</sub>O, 10% (w/v) sucrose, and 4% (v/v) or 10% (v/v) glycerol. The active PFK species sediments as a single component with a sedimentation coefficient of  $12.4 \pm 0.5$  S, after correcting for protein-solvent interactions. Although PFK may undergo association-dissociation, there is no observable change in the value of  $s_{20,w}$  over a 57-fold range of protein concentration. Throughout this range only a single active species of PFK was observed, and within an experimental uncertainty of  $\pm 10\%$ , the enzymatic activity observed in the sedimentation studies

accounts for the total enzymatic activity observed in the steady-state kinetics. Partially purified PFK was subjected to AEC analysis. Results reveal the presence of again a single active form sedimenting at the same rate as the purified enzyme. Results from sedimentation velocity studies indicate that the stabilizing solvents employed in AEC enhance the self-association of PFK. However, such an enhancement alone cannot account for the observation of a single active species with a sedimentation coefficient of 12.4 S. The interactions between solvent additives and PFK were studied by density measurements and by the application of multicomponent theory. Results from such a preferential solvent interaction study indicate that PFK is preferentially hydrated in the presence of sucrose or glycerol. The enhancement of PFK self-association is most likely due to a nonspecific solvent-protein interaction.

**P**hosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) catalyzes the transfer of the terminal phosphate of ATP to the C-1 hydroxy of fructose 6-phosphate to produce fructose 1,6-diphosphate. This reaction

represents a key control point in glycolysis. As a result of extensive investigations (Ling et al., 1965; Parmeggiani et al., 1966; Aaronson & Frieden, 1972; Pavelich & Hammes, 1973; Lad & Hammes, 1974; Leonard & Walker, 1972), it has been demonstrated clearly that rabbit muscle phosphofructokinase (PFK) is a complex regulatory enzyme capable of existing in a variety of polymeric forms.

Hammes and co-workers (Pavelich & Hammes, 1973; Parr & Hammes, 1975, 1976; Hill & Hammes, 1975; Lad et al., 1973; Lad & Hammes, 1974), in a series of reports on the

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states of PFK aggregation studied by frontal gel chromatography, demonstrated that at concentrations below 0.2 mg/mL PFK undergoes a concentration-dependent reaction involving a dimer and a tetramer. Strong activators such as fructose phosphates stabilize the tetramer while inhibitor favors the smaller aggregates. The rates of association-dissociation were rapid (with half-times of <5 min) in the presence of effectors at 5 °C. However, the rates become slower (with half-times of 10–40 min, depending on the pH) without effectors. Conceivably, in the presence of substrates and effectors and at higher temperature the rate of reaction may be much faster. In subsequent publications, Parr & Hammes (1975, 1976) reported on kinetic studies of subunit dissociation of PFK. It was shown that the reassembly of PFK from its monomeric subunits is enhanced by the presence of ATP and the dissociation of tetramer to dimer is characterized by a relaxation time of a few milliseconds. Furthermore, Lad & Hammes (1974) using dimethyl suberimidate as the cross-linking reagent have demonstrated that the dimer is a fundamental unit for polymerization of the enzyme. It is evident that the equilibrium between various states of aggregation can be perturbed by the presence of ligands. It leads to the question, then, "What is the state of aggregation that is enzymatically active?"

Although there are reports indicating that aggregates larger than tetramer are active and have the same specific activity as tetramer (Aaronson & Frieden, 1972) and that dimer or monomers are not active or have lower specific activity (Pavelich & Hammes, 1973), all of these studies suffer in varying degrees from technical difficulties. The identity of the active species and the answer to the question "How is the activity of PFK regulated by the changing of quaternary structure?" have not been proven unequivocally. For investigation of the identity of active enzyme-substrate complexes, density gradient centrifugation (Wenzel et al., 1972) and gel filtration (Pavelich & Hammes, 1973; Aaronson & Frieden, 1972; Hofmann et al., 1975) would not be the methods of choice because these methods rely on a physical separation of the different aggregated forms of the enzyme and a subsequent test for catalytic activity. Such procedures cannot remove uncertainties concerning possible changes in the aggregation state after separation is completed or in the assay mixture.

The reacting enzyme sedimentation technique (Cohen et al., 1967; Cohen & Mire, 1971) employed in the present study, however, will overcome these technical difficulties. The advantages of the method are as follows: (1) the hydrodynamic properties of the enzyme-substrate complex can be determined while it is fully active; hence, any difference in polymerization state between the free and the reacting enzyme can be directly determined; (2) sedimentation can be observed at the very dilute enzyme concentrations used in kinetic studies; therefore, the determined hydrodynamic properties can be directly compared with the kinetic data. A preliminary sedimentation study having indicated that the predominantly active form of PFK sediments with  $s_{20,w} = 12.3$  S (Hesterberg & Lee, 1979), a detailed study of the active form of PFK was initiated and the results are reported in this paper.

#### Materials and Methods

Rabbit muscle phosphofructokinase, aldolase, triosephosphate isomerase-glycero-3-phosphate dehydrogenase, ATP, NADH, DTT, fructose 6-phosphate, and D<sub>2</sub>O were obtained from Sigma Chemical Co. Glycerol and cresol red were purchased from Fisher Scientific Co. Ultrapure sucrose was from Schwarz/Mann. All enzymes and chemicals were

used without further purifications except phosphofructokinase.

Rabbit muscle PFK was purified by using the method of Hussey et al. (1977), followed by ion-exchange chromatography as outlined by Uyeda et al. (1978). Following the final DEAE column, PFK fractions with a specific activity of >200 units/mg were pooled. The enzyme was stored as a suspension in 80% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. NaDodSO<sub>4</sub>-polyacrylamide gel electrophoresis (Laemmli, 1970) was employed to assess the homogeneity of the enzyme at various stages of the purification scheme. The purified PFK migrated as a single band, indicating >98% homogeneity. Identical results were obtained regardless of the source of the enzyme, be it commercial or purified in this laboratory.

Activity of the PFK at pH 8.55 was monitored by two methods: a modified coupled enzyme system (Racker, 1947) and a pH-dependent, dye-linked assay system (Shill et al., 1974). In both systems glycylglycine was the buffer of choice. The standard coupled enzyme system contained 25 mM glycylglycine, 1.0 mM EDTA, 3.4 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 6.0 mM MgCl<sub>2</sub>, 0.1 mM DTT, 1 mM fructose 6-phosphate, 1 mM ATP, 0.16 mM NADH, 1.25 units/mL aldolase, and 2 units/mL mixed enzymes glycero-3-phosphate dehydrogenase-triosephosphate isomerase with the pH adjusted to 8.55 ± 0.05. The auxiliary enzymes were used without further purification. The enzymes were passed over a Sephadex G-25 desalting column (0.9 × 4 cm) equilibrated with 25 mM glycylglycine, 1 mM EDTA, 6 mM MgCl<sub>2</sub>, and 3 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> at pH 8.55 (GEMA buffer). The reaction was initiated by the addition of 50 µL of a desired concentration of PFK to a volume of 1.45 mL of assay mixture and monitored continuously at 340 nm on a Cary-118 recording spectrophotometer. The specific activity of the PFK used was never lower than 190 units/mg.

The standard pH-dependent dye-linked assay system (Shill et al., 1974) contained 1.65 µM glycylglycine, 5 µM MgCl<sub>2</sub>, 4.5 µM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 mM ATP, 1 mM fructose 6-phosphate, and 33 µg/mL cresol red at pH 8.55 ± 0.05. The reaction was initiated by the addition of 50 µL of PFK to a volume of 1.45 mL of assay mixture and monitored continuously at 560 nm. Titration of the dye assay mixture at pH 8.55 by the addition of HCl revealed a nonlinear relation between absorptivity and titrant added. Such nonlinear behavior has been reported by Shill et al. (1974). The region of interest, however, was within the initial linear range between 0 and 10 nmol of H<sup>+</sup> with a change of  $1.5 \times 10^{-3}$  in absorptivity at 560 nm per nmol of H<sup>+</sup> ion.

Protein concentrations were determined by absorbance at 280 nm with an absorptivity of 1.07 L/(g cm) in glycylglycine buffer at pH 8.55. The extinction coefficient was determined by the differential refractometry method of Babul & Stellwagen (1969). Fringe displacements were measured on a Nikon 6C microcomparator. For all experiments, an aluminum-filled Epon synthetic boundary centerpiece was used, and bovine serum albumin was employed as the standard. As a control, solutions of egg white lysozyme in the same buffer were subjected to analysis. An absorptivity value of 2.64 L/(g cm) at 282 nm was obtained, agreeing favorably with the literature value of 2.635 L/(g cm) (Sophianopoulos et al., 1962). Solutions of PFK in the GEMA buffer with known absorbance at 280 nm were subjected to sedimentation analysis. After determination of the fringe displacements, concentrations of these solutions were then obtained from the standard graph and an absorptivity value of  $1.07 \pm 0.02$  was obtained. Extinction coefficients of 1.07 and 1.14 L/(g cm) were determined for PFK in 10% (w/v) sucrose and 10% (v/v)

glycerol, respectively, by the method of difference spectroscopy.

All sedimentation studies were carried out with a Beckman-Spinco Model E analytical centrifuge equipped with a UV scanner. The temperature was regulated and measured by the RTIC unit. For boundary sedimentation velocity studies, 12-mm path length Kel-F-coated aluminum double-sector centerpieces with sapphire windows were used in an AN-D rotor at 60000 rpm. The boundary was monitored with the UV scanner at 237 nm. For reacting enzyme sedimentation studies, a charcoal-filled Epon 12-mm path length, Type I double-sector band forming centerpiece with sapphire windows was used in an AN-D rotor at 60000 rpm. Both sectors were filled with 0.32 mL of the respective assay mixture containing either 50% (v/v) D<sub>2</sub>O, 10% (v/v) glycerol, or 10% (w/v) sucrose as a stabilizing gradient. The capillary chamber of the sample sector was filled with 10  $\mu$ L of assay buffer containing the appropriate concentration of PFK minus the substrates. The chamber of the reference sector was filled with 10  $\mu$ L of assay buffer.

A UV scanner was used to measure the reaction products formed during centrifugation of the band of enzyme through the assay mixture. Sedimentation coefficients were determined from the midpoints of the scanner-traced boundaries and checked against the sedimentation velocity calculated from the line of symmetry of the difference curves (Cohen et al., 1967). The tracings were obtained at the fastest scan speed and 25 mm/s chart speed at either 2- or 4-min intervals. Observed  $s$  values were obtained with a least-squares analysis of the  $\ln R$  vs. time plots.

The observed sedimentation coefficients were normalized to standard conditions by correcting for solvent density, viscosity, and preferential solvent interactions in various solvent systems in accordance to (Schachman, 1959)

$$s_{20,w} = s_{\text{obsd}} \frac{\eta_{t,w}}{\eta_{20,w}} \frac{\eta_{\text{solvent}}}{\eta_{t,w}} \frac{(1 - \bar{v}^{\circ}_2 \rho_w)_{20}}{(1 - \phi^{\circ}_2 \rho_{\text{solvent}})_{20}} \quad (1)$$

where  $\eta$  is viscosity,  $\rho_w$  and  $\rho_{\text{solvent}}$  are the densities of water and the solvent, respectively, and  $\bar{v}^{\circ}_2$  and  $\phi^{\circ}_2$  are partial specific volumes of the protein in water and in the presence of organic solvents, respectively. The observed sedimentation coefficients in the presence of 50% (v/v) D<sub>2</sub>O were corrected for deuteration of protein in addition to solvent density and viscosity by using the procedures outlined by Taylor et al. (1972).

Viscosities of the assay mixtures in the presence of organic solvents were determined at  $20.00 \pm 0.01$  °C with an Oswald-type semimicroviscometer with a flow time of 136.8 s for distilled deionized water.

The partial specific volume,  $\bar{v}^{\circ}_2$ , of PFK in GEMA buffer and the apparent partial specific volume,  $\phi^{\circ}_2$ , of the protein in 10% (w/v) sucrose and 4 and 10% (v/v) glycerol were obtained by measuring the densities of protein solutions of various concentrations and those respective solvents against which they had been dialyzed for 18–24 h at 4 °C. The densities were measured with a precision density meter (Mettler/Paar DMA 02D) at  $20.00 \pm 0.01$  °C. The concentration and density data were combined to obtain the apparent values of  $\bar{v}_2$  or  $\phi_2$  at each protein concentration,  $c$ , according to

$$\phi_c = \frac{1}{\rho_0} \left( 1 - \frac{\rho - \rho_0}{c} \right) \quad (2)$$

where  $\rho_0$  is the solvent density,  $\rho$  is the protein solution density, and  $c$  is the protein concentration in g/mL. Values of  $\bar{v}^{\circ}_2$  and

$\phi^{\circ}_2$  were obtained by extrapolation of the apparent values to zero protein concentration.

The partial specific volumes obtained were used in the calculation of  $s_{20,w}$  from the sedimentation velocity data.

For determination of the activity of the enzyme during active enzyme sedimentation and for comparison of this activity to the steady-state kinetic activity, difference curves were constructed as outlined by Cohen & Claverie (1975). The area under each curve in cm<sup>2</sup> was determined by the product of half its base times the height (in cm) of each curve at its peak. The observed base and height of the difference curve must be corrected for the intrinsic magnification factor, calibration of absorptivity vs. pen deflection, and path length in the centrifuge cell. The area of the triangle actually represents the total change in absorption for the time period between scans. The enzyme activity expressed as the change in absorption per minute can be calculated by dividing the total area calculated for a given difference curve by the number of minutes between the two successive scans. It is, therefore, possible to compare the enzyme activity observed in the active enzyme sedimentation experiment with that determined by steady-state kinetics.

## Results

Most of the reports on PFK in the literature include phosphate in their buffer systems in order to stabilize the enzyme (Leonard & Walker, 1972; Paradies & Vettermann, 1976; Bloxham & Lardy, 1973; Jones et al., 1975; Kee & Griffin, 1972; Pavelich & Hammes, 1973; Goldhammer & Hammes, 1978; Pettigrew & Frieden, 1979). It has been shown at least at pH 7.1, however, that the kinetic properties of the enzyme depend on the identity of the buffer (Hofer & Pette, 1968). In the presence of phosphate buffer, PFK exhibited a hyperbolic relation between relative specific activity and protein concentration, whereas in the presence of glycylglycine buffer such relation was altered and became sigmoidal. When the mode of regulation on PFK activity was explored, the condition chosen, therefore, was that which might amplify its regulatory behavior, i.e., glycylglycine buffer. Since phosphate is omitted from the present study, it is imperative to ascertain the stability of the enzyme in glycylglycine buffer, enabling a direct correlation between steady-state kinetic observations and physical measurements which may take hours to complete. The stability of PFK under conditions similar to those used in the sedimentation and kinetic studies was investigated.

PFK was incubated at 23 °C and at protein concentrations of 60 and 120  $\mu$ g/mL in GEMA buffer with 0.1 mM DTT. The effects of 10% (w/v) sucrose, 10% (v/v) glycerol, and 50% (v/v) D<sub>2</sub>O on PFK stability were also studied by incorporating these solvents into the incubation buffer. No substrates or effectors were present. At a given time after the incubation was started, aliquots of 50  $\mu$ L were removed and the enzyme activity was monitored by the coupled enzyme assay system. Control experiments were conducted to determine the effect of 50  $\mu$ L of 10% (w/v) sucrose, 10% (v/v) glycerol, or 50% (v/v) D<sub>2</sub>O on the observed activity, and the results showed that the presence of 50  $\mu$ L of any of these solvents exhibited no observable effects on the activity. Thus, the activity observed in the presence of these solvents is then directly comparable to that in the absence of these solvents. The activity observed as a function of time was plotted as the percent of the initial activity as shown in Figure 1. The initial specific activity of PFK was 190 units/mg. The stability of PFK under the present experimental conditions is apparently not affected by the protein concentration employed in this study since a

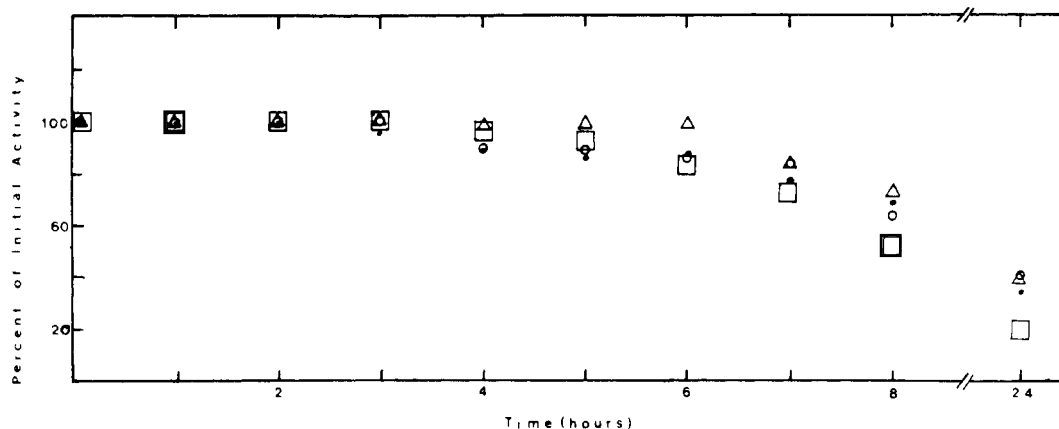


FIGURE 1: Stability of PFK. The enzyme concentration was 60  $\mu\text{g}/\text{mL}$  with a specific activity of 190 units/mg. The buffer was 25 mM glycylglycine, 1.0 mM EDTA, 3.4 mM  $(\text{NH}_4)_2\text{SO}_4$ , 6.0 mM  $\text{MgCl}_2$ , and 0.1 mM DTT. The symbols and solvent additives are (□) no additive, (●) 50% (v/v)  $\text{D}_2\text{O}$ , (○) 10% (v/v) glycerol, and (Δ) 10% (w/v) sucrose.

Table I: Summary of  $K_m$  Values of PFK in Various Solvents

buffer	solvent	$K_m$		
		ATP ( $\mu\text{M}$ )	F-6-P ( $\mu\text{M}$ )	ITP (mM)
GEMA <sup>a</sup>		40 $\pm$ 5	50 $\pm$ 5	0.13 $\pm$ 0.01
GEMA	50% (v/v) $\text{D}_2\text{O}$	37 $\pm$ 5		
GEMA	10% (w/v) sucrose	45 $\pm$ 5		
GEMA	10% (v/v) glycerol	40 $\pm$ 5		
$\text{PO}_4$		61 $\pm$ 5 <sup>b</sup>	44 $\pm$ 2 <sup>b</sup>	
$\text{PO}_4$		75 <sup>c</sup>		
Tris-HCl		20 <sup>d</sup>	21 <sup>d</sup>	
triethanolamine hydrochloride			200 <sup>e</sup>	7.0 <sup>e</sup>

<sup>a</sup> Buffer consists of 25 mM glycylglycine, 1.0 mM EDTA, 6.0 mM  $\text{MgCl}_2$ , 3.4 mM  $(\text{NH}_4)_2\text{SO}_4$ , and 0.10 mM DTT, pH 8.55.

<sup>b</sup> Kee & Griffen (1972). <sup>c</sup> Kühn et al. (1974). <sup>d</sup> Hanson et al. (1973). <sup>e</sup> Uyeda (1970).

twofold difference in the initial protein concentration did not affect the relative specific activity. These studies show that PFK used in these studies remains viable with less than a 15% decrease in activity after 6 h. Since most of the sedimentation experiments were completed within 4–5 h and in no circumstances were experiments conducted with enzyme that was kept at room temperature beyond 6 h, the results from this study, therefore, are conformed to PFK that retains at least 85% of its initial activity.

A change in the buffer system may induce PFK to exhibit different kinetic behavior. A study was, therefore, carried out to compare the basic steady-state kinetics of PFK in glycylglycine buffer at pH 8.55. The data were analyzed according to Lineweaver & Burk (1934), and the results are summarized in Table I. It is evident that at least the values for  $K_m$ , the Michaelis constant, are consistent with the literature values. It may be concluded that under the present experimental conditions PFK does not exhibit any different kinetic behavior based on its affinity for substrates with no indications of cooperativity in substrate binding.

One of the requirements for active enzyme centrifugation experiments is an adequate positive density gradient in the assay mixture to counteract the negative gradient associated with the leading edge of the sedimenting band. Sucrose, glycerol, and  $\text{D}_2\text{O}$  are the solvents employed in this study to generate the gradient. Studies were carried out to determine the effects of these solvents on the kinetic behavior of PFK.

As shown in Table I, within experimental uncertainties, the presence of these solvents does not alter the  $K_m$  values for ATP. Furthermore, it was shown that cresol red has no observable effect on PFK activity under the assay conditions employed. It may be concluded that these solvents and dye do not induce significant alterations in the kinetic behavior of PFK and the results from sedimentation experiments most probably reflect the actual state of PFK in the presence of substrates.

Active enzyme centrifugation was employed to identify the enzymatic active component of PFK. Such a study was conducted by utilizing two enzyme assay systems, namely, coupled enzyme and pH dye-linked systems. Three different solvents were used to supply the required stabilizing gradient. The coupled enzyme system is more sensitive than the pH dye-linked system; hence, a wider range of PFK concentration could be examined by utilizing both systems although each system suffers from some basic limitations which will be discussed later. The rationale for employing three solvent systems was to circumvent or sort out the possibility of non-specific solvent-protein interactions which might influence the results so that they do not reflect the behavior of PFK in solution in the absence of these solvents.

Figure 2A is a composite image of scanner tracings at 340 nm of the sedimentation of 7.5  $\mu\text{L}$  of 3.15  $\mu\text{g}/\text{mL}$  PFK in the coupled enzyme system with 50% (v/v)  $\text{D}_2\text{O}$  as the stabilizing solvent. It is evident that the tracings indicate the presence of apparently one detectable sedimenting component under the present experimental conditions. Sedimentation coefficients were determined from the midpoints of the scanner-traced boundaries. Figure 2B shows the relationship between  $\ln R$  and time, where  $R$  is the radius of rotation. An apparent value of  $s_{20,w} = 12.7 \text{ S}$  was calculated. Cohen and co-workers (Cohen & Mire, 1971; Cohen & Claverie, 1975) discussed the advantages of measuring  $s_{20,w}$  of active enzyme by expressing the data as the difference distribution of product at each point  $R$  and plotting the logarithm of the position of the band maximum vs. time. Accordingly, a difference curve is produced for each pair of consecutive scans as shown in Figure 2C. The relation between the logarithms of the position of the band maximum and time was shown in Figure 2B. The data points obtained are in good agreement with those monitored by the midpoints of the sedimenting boundaries.

The difference curves correspond to the distribution of enzyme activity and therefore represent the shape and average position of the reacting enzyme band as it sediments through the cell. The difference curves as shown in Figure 2C indicate the sedimentation of a symmetric reacting enzyme band with

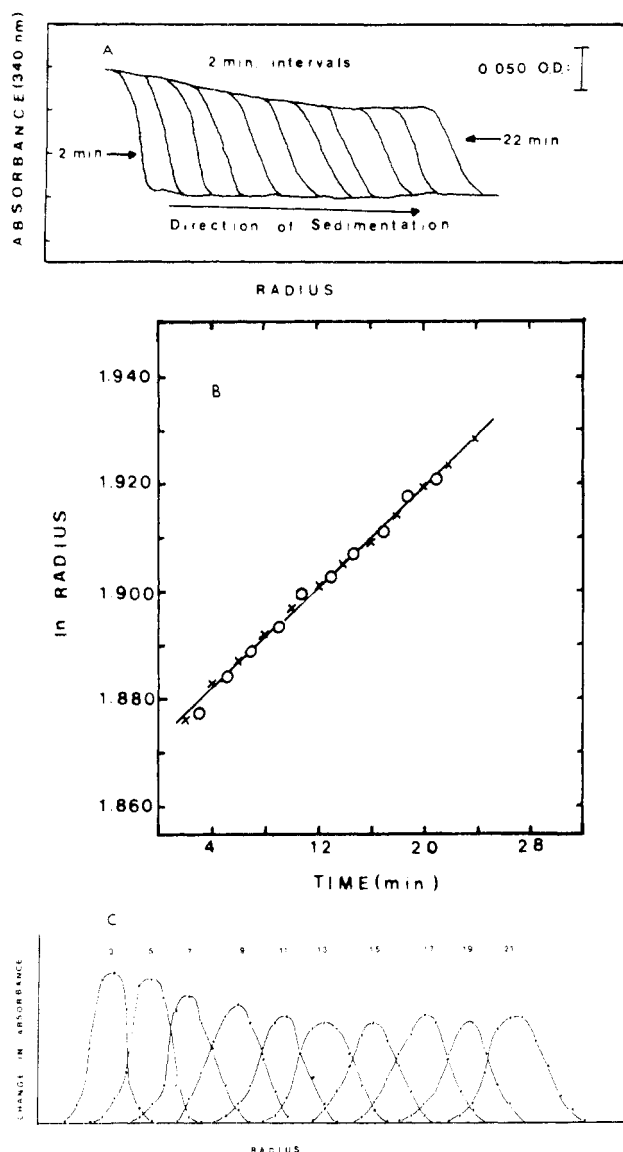


FIGURE 2: Active enzyme sedimentation of PFK at pH 8.55 and 23 °C. The coupled enzyme assay system was utilized in 50% D<sub>2</sub>O. The conditions for centrifugation were as follows: rotor speed, 60000 rpm; scan speed, fastest; chart speed, 25 mm/s; noise suppression, off; wavelength, 340 nm; optical density range, 0–1.0; 7.5  $\mu$ L of PFK at 3.15  $\mu$ g/mL was layered onto the assay mixture. (A) Successive scanner tracings superimposed on a single frame of reference. (B) Relation between  $\ln R$  and time for data obtained from the inflection point of the boundary ( $\times$ ) and the difference curve ( $\circ$ ). The time of the difference curve was taken to be the mean time of the scans. (C) Difference curves constructed from the scanner traces.

no consistent skewness. Such observation implies the presence of a homogeneous active species.

One of the major concerns in active enzyme sedimentation experiments is denaturation of the enzyme in the course of these experiments. Since the area enclosed by the sedimentation boundary represents the amount of product in the cell, it is possible to monitor the activity of PFK in the course of a sedimentation experiment. If no denaturation of PFK or no dissociation of PFK into inactive smaller aggregates during the sedimentation procedure has occurred, then the total amount of product in the cell should increase linearly with time. Accordingly, the total amount of product, expressed in arbitrary units of area, is plotted as a function of time of sedimentation as shown in Figure 3. A linear relation is established for PFK under the present experimental conditions. Evidently there is no loss of activity due to denaturation nor

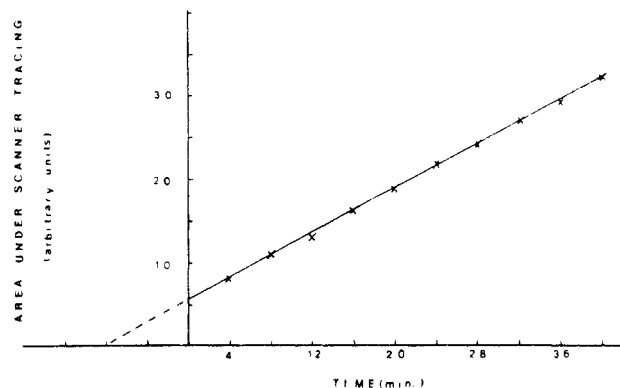


FIGURE 3: Amount of product formed and time of sedimentation. Enzyme activity is assumed to be proportional to the area enclosed by the scanner tracing. The time shown is from the start of scans, while the actual layering time, as observed visually, occurred 10.75 min prior to the initial scan at 0 min.

Table II: Comparison of Phosphofructokinase Activity: Steady-State Kinetics vs. Active Enzyme Sedimentation

assay system	stabilizing gradient solvent	obsd av $\Delta OD/\text{min}^a$	
		steady state	AEC
coupled enzyme	50% D <sub>2</sub> O	$5.1 \times 10^{-2}$	$5.3 \times 10^{-2}$
coupled enzyme	10% sucrose	$2.3 \times 10^{-1}$	$2.1 \times 10^{-1}$
coupled enzyme	10% glycerol	$2.4 \times 10^{-1}$	$2.5 \times 10^{-1}$
pH dye	10% glycerol	$9.2 \times 10^{-3}$	$8.2 \times 10^{-3}$

<sup>a</sup> Observed  $\Delta OD$  is normalized to 1  $\mu$ g of PFK, 1-mL volume, and 1.0-cm path length.

dissociation into an inactive form of the enzyme in the course of these experiments.

The area under each difference curve represents the total activity of the enzyme for the time between two consecutive scans. It is, therefore, possible to estimate quantitatively the activity of PFK during the centrifugation experiment, enabling a correlation to be made to the activity observed in the steady-state kinetic studies conducted in identical solutions and at the same temperature. As the exact amount of PFK present at any point in the centrifuge cell was not known, the activity was calculated by assuming that all of the protein added to the capillary chamber was layered and sedimenting in the cell. The results of such calculations were summarized in Table II. It is evident that the corrected activity observed per minute was in good agreement with the kinetic data. One may then conclude that within the experimental uncertainty of  $\pm 10\%$  all of the activity observed in the kinetic study was accounted for in the active enzyme sedimentation experiment.

Active enzyme sedimentation of PFK was also carried out by using 4% (v/v) or 10% (v/v) glycerol and 10% (w/v) sucrose as stabilizing gradients. Sedimentation boundaries similar to those in 50% D<sub>2</sub>O (Figure 2A) were observed. Furthermore, difference curves computed the same as those in the presence of D<sub>2</sub>O indicate also symmetrical peaks in either solvent system, demonstrating the presence of a homogeneous active species. The relations between  $\ln R$  and time, regardless of  $R$  being computed from the midpoint or the difference plot, were linear, indicating the absence of detectable denaturation or dissociation of PFK within the time interval of observation. Values of  $11.5 \pm 0.2$ ,  $10.6 \pm 0.3$ , and  $10.3 \pm 0.4$  S were calculated for  $s_{20,w}$  in 4% (v/v) glycerol, 10% (v/v) glycerol, and 10% (w/v) sucrose, respectively. These values represent the mean and standard error of repetitive experiments and were obtained after correcting for

Table III: Preferential Interactions of Solvent Components with Phosphofructokinase

stabilizing solvent (component 3)	$\phi^{\circ}_2$ (mL/g)	$\bar{v}_2$ (mL/g)	$\frac{\partial g_3}{\partial g_2}$ (g/g)	$\frac{\partial m_3}{\partial m_2^a}$ (mol/mol)
		$0.730 \pm 0.008^b$		
10% (w/v) sucrose	$0.761 \pm 0.004$		-0.032	-43
4% (v/v) glycerol	$0.742 \pm 0.004$		-0.012	-43
10% (v/v) glycerol	$0.753 \pm 0.002$		-0.024	-84

<sup>a</sup> Assumes a tetramer of 320 000. <sup>b</sup> It is assumed that the apparent partial specific volume at constant chemical composition has a value identical with that of  $\bar{v}_2$ .

solvent density and viscosity and assuming  $\phi^{\circ}_2 = \bar{v}^{\circ}_2$ . Intuitively it may be concluded that in the presence of these solvents the active form of PFK is smaller than that in  $D_2O$ . However, such an apparent decrease in sedimentation coefficient may be a consequence of nonspecific thermodynamic interaction between proteins and organic solvents. Such interaction is reflected in a change in partial specific volume and would lead to dramatic changes in the value of  $s_{20,w}$  as shown by eq 1. Timasheff and co-workers (Lee et al., 1979; Lee & Timasheff, 1977; Timasheff et al., 1976b) have demonstrated that proteins in sucrose and glycerol are preferentially hydrated. A solvent-protein interaction study was, therefore, initiated to investigate the effect of these organic solvents on PFK. The results of this study are summarized in Table III. The apparent partial specific volume in the presence of solvent additives assumed higher values than that in the absence of these solvents, indicating a preferential hydration of PFK. After determination of the apparent partial specific volume of PFK in sucrose and glycerol, these values can be substituted into eq 1 to calculate for a corrected value of  $s_{20,w}$ , yielding  $s_{20,w} = 12.8, 12.2$ , and  $12.4$  S for the active component of PFK in sucrose, 4% glycerol, and 10% glycerol, respectively. It may be concluded that the active form of PFK, as determined in the presence of three different solvents and utilizing the coupled enzyme system, is sedimenting as a single component with an average  $s_{20,w}$  of  $12.5 \pm 0.5$  S.

One of the prerequisite conditions of active enzyme sedimentation is that the coupled enzymes should have a smaller  $s_{20,w}$  than the enzyme of interest. If PFK's were active as components much smaller than 12 S, the coupled enzyme system may not be capable of detecting their presence, since the auxiliary enzymes, namely, aldolase and the  $\alpha$ -glycero-3-phosphate dehydrogenase-triosephosphate isomerase complex, sediment with  $s_{20,w}$  values of 8.4 and 5 S, respectively. The dimeric and monomeric forms of PFK are expected to sediment as 7.6S and 4.8S particles, and these components are much smaller than the coupled enzymes. To circumvent such limitations, the pH-dependent dye-linked system was employed by using 10% (v/v) glycerol as the stabilizing gradient. In such an assay system, PFK is the only protein present. The results showed that one sedimentation boundary is observed under the present experimental conditions, an observation that is consistent with the results using the coupled enzyme assay system. The difference curves computed showed symmetric peaks throughout the time interval of the experiment. Within experimental uncertainties, there was no indication of skewed peaks. The relations between  $\ln R$  and time, regardless of  $R$  being computed from the midpoint or the difference plot, were linear and yielded a  $s_{20,w} = 12.2 \pm 0.3$  S. Again, computation of enzyme activity based on the difference curves revealed that

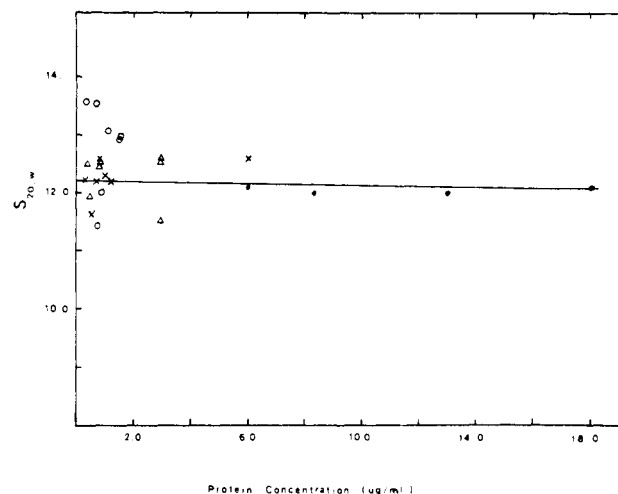


FIGURE 4: Relation of  $s_{20,w}$  obtained from active enzyme sedimentation experiments and PFK concentration. The symbols and experimental conditions are (X) coupled enzyme assay in 10% (v/v) glycerol, (O) coupled enzyme assay in 10% (w/v) sucrose, ( $\Delta$ ) coupled enzyme assay in 50%  $D_2O$ , and ( $\bullet$ ) pH-dependent dye-linked assay in 10% (v/v) glycerol.

the enzyme activity as monitored by steady-state kinetics can be accounted for in the centrifugation experiment as shown in Table II. It may be concluded that within the limits of resolution of the technique and experimental conditions employed the enzymatic active species of rabbit muscle PFK sediments as a single component with a sedimentation coefficient of  $12.4 \pm 0.5$  S.

PFK is known to undergo association-dissociation into various oligomeric forms (Parmeggiani et al., 1966; Leonard & Walker, 1972; Aaronson & Frieden, 1972; Pavelich & Hammes, 1973). It is conceivable that at different ranges of protein concentrations such forms might be present in a significant amount in the assay mixture and might also be active. The identity of the enzymatically active form of PFK was monitored as a function of protein concentration, utilizing both the coupled enzyme and pH dye-linked assay systems, to search for the presence of such active oligomers. Results of such studies are shown in Figure 4. It is evident that within the concentration range of 0.3–18  $\mu\text{g/mL}$  there is no apparent change in the value for  $s_{20,w}$ , indicating that there are no detectable active PFK components other than that with  $s_{20,w}^0$  of 12.4 S. It is possible, however, that at higher protein concentrations larger active forms might be present in a significant amount. Under the present experimental conditions, the 12S component is apparently the predominant, if not the only, active form of PFK.

Cohen & Mire (1971) showed that an apparent increase of  $s_{20,w}$  would be observed if there were significant depletion of substrates. The fact that the values of  $s_{20,w}$  did not increase with protein concentration is an indication that most likely there is no significant depletion of substrates. In order to substantiate such a conclusion, active enzyme sedimentation experiments were carried out as a function of substrate concentrations. ATP concentration was maintained at 1 mM in order to circumvent its inhibitory effect at high concentration. At higher concentrations of substrates, ITP was added to 1 mM ATP. The value of  $s_{20,w}$  did not change as a function of total substrate concentration from 1 to 5 mM. Theory predicts, however, that if substrate depletion occurs during the passage of the band of enzyme solution, the value of  $s_{20,w}$  would decrease with higher substrate concentration. Such substrate depletion may be brought about by either overloading the system with enzyme or insufficient substrate was included in

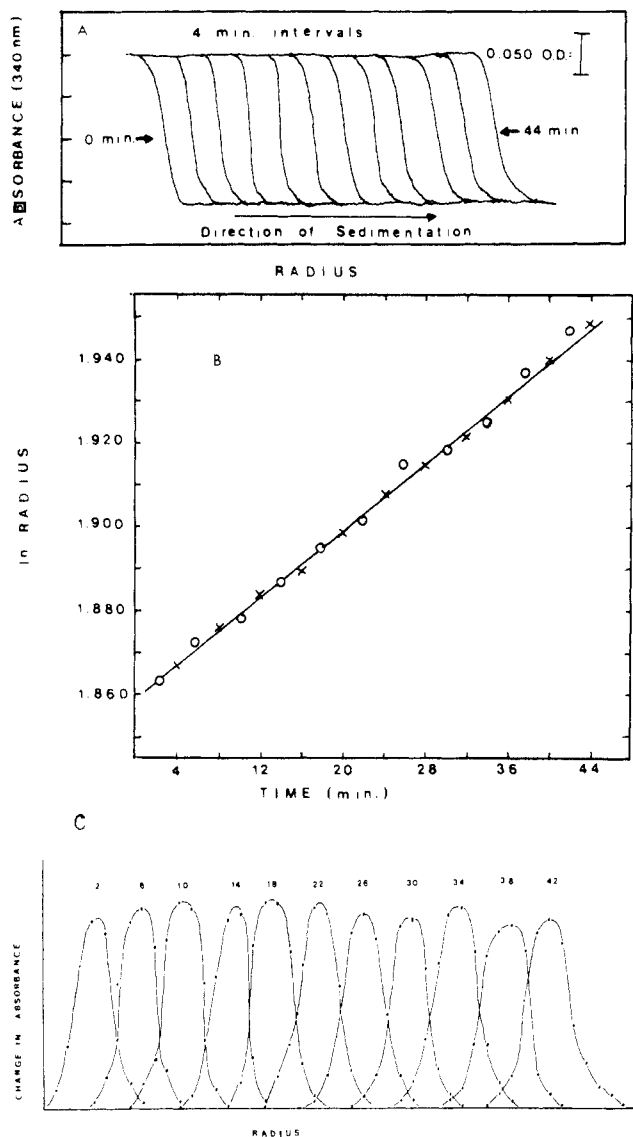


FIGURE 5: Active enzyme sedimentation of rabbit muscle homogenate at pH 8.55 and 23 °C. The coupled enzyme assay system was utilized in 10% (v/v) glycerol. The experimental conditions are the same as those described in Figure 2 except that 10  $\mu$ L of PFK at a specific activity of 30 units/mg and 160  $\mu$ g/mL was layered onto the assay mixture. (A) Successive scanner tracings superimposed on a single frame of reference. (B) Relation between  $\ln R$  and time for data obtained from the inflection point of the boundary (x) and the difference curve (o). The time of the difference curve was taken to be the mean time of the scans. (C) Difference curves constructed from the scanner traces.

the assay mixture. On the basis of the evidences presented, namely, no dependence in  $s_{20,w}$  as a function of enzyme concentration studied and no change in  $s_{20,w}$  with increasing substrate concentrations, it may be concluded that under our present experimental conditions sufficient substrate was present even for the highest PFK concentration tested. Furthermore, the absence of detectable smaller active species is most likely not due to the lack of substrate after the passage of the larger one.

An advantage of active enzyme sedimentation is that it is possible to monitor the active component of the enzyme in an impure state. Such advantage was taken to explore the active form of PFK in a crude homogenate. A sample of PFK after the initial  $(\text{NH}_4)_2\text{SO}_4$  precipitation was subjected to sedimentation analysis, results of which are shown in Figure 6. One sedimentation boundary was observed with an apparent

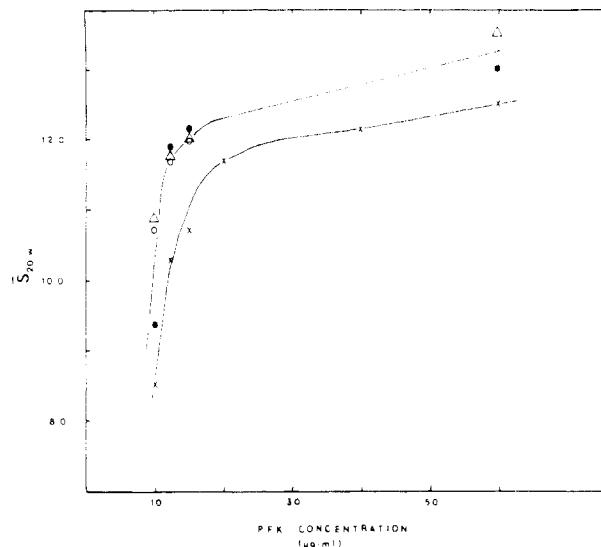


FIGURE 6: Relation between the weight-average sedimentation coefficient,  $s_{20,w}$ , and PFK concentration at pH 8.55 and 23 °C in GEMA buffer in the absence of substrates. The symbols and solvent additives are (x) none, (o) 10% (v/v) glycerol, ( $\Delta$ ) 10% (w/v) sucrose, and ( $\bullet$ ) 50%  $\text{D}_2\text{O}$ .

$s_{20,w}$  of  $12.5 \pm 0.4$  S. More interestingly, the difference curves calculated, as shown in Figure 5C, show the presence of symmetrical sedimenting peaks with no indication of skewness during the total duration of the sedimentation experiment. The apparent  $s_{20,w}$  is in good agreement with those observed utilizing highly purified PFK samples. Such observation implies that PFK, at least for the enzyme that has been partially purified and under our present experimental conditions, behaves as an independent component. It is not associated with other cellular components forming a complex. It is conceivable, however, that under more physiological conditions and in an unperturbed state in the cell, PFK may yet exist in forms other than that shown in Figure 5.

The results from active enzyme sedimentation experiments indicate that in the presence of a full complement of substrates rabbit muscle PFK sediments as a 12S component. It is conceivable that the enzyme exists in other oligomeric forms in the absence of substrates. There is evidence in the literature (Pavelich & Hammes, 1973) that at low concentrations of PFK the enzyme exists as a mixture of dimeric and tetrameric forms, the equilibrium of which can be perturbed by metabolites (Lad et al., 1973). A preliminary study was initiated to study the sedimentation behavior of PFK at low concentration in the absence of ligands. The concentration range of PFK studied was 10–60  $\mu$ g/mL, and the observed  $s_{20,w}$  values are summarized in Figure 6. It is evident that the observed value for  $s_{20,w}$  decreases from 11.3 to 8.5 S with decreasing concentration of PFK. The extrapolated value of  $s_{20,w}$  at protein concentrations equivalent to those employed in active enzyme sedimentation is much below 12 S. Such a decrease in  $s_{20,w}$  indicates a dissociation of PFK into smaller components under these conditions, a conclusion that is consistent with reports by Pavelich & Hammes (1973). The presence of substrates may then perturb the equilibrium so as to favor the formation of larger aggregates as indicated by the larger value of  $s_{20,w}$  for the active form. It is conceivable, however, that the presence of solvent components employed in this study may also perturb the equilibrium of PFK association–dissociation. The sedimentation behavior of PFK in the presence of these solvents at low protein concentrations was monitored, and the results are summarized in Figure 6. In all solvents tested, the observed values for  $s_{20,w}$  at equivalent protein concentrations



are higher than that in the absence of the organic solvents and  $D_2O$ . It implied that these solvents apparently perturbed the equilibrium of PFK self-association, favoring the higher aggregate. However, in all solvents tested the general trend of dissociation into smaller aggregates at lower protein concentration was still maintained, and the extrapolated value of  $s_{20,w}$  at concentrations employed in active enzyme sedimentation is still much lower than 12 S. It may be concluded that these solvents enhance the formation of the larger oligomeric form(s) of PFK but probably do not alter the mode of self-association of the enzyme.

### Discussion

The enzymatic active form of rabbit muscle PFK has been the subject of intensive investigations (Wenzel et al., 1972; Pavelich & Hammes, 1973; Aarsonson & Frieden, 1972; Hofmann et al., 1975; Paetkau & Lardy, 1967; Bock & Frieden, 1974). A direct demonstration of the aggregation state of PFK while it is enzymatically active, however, has not been reported although studies on erythrocyte and yeast PFK were reported (Karadsheh et al., 1977; Kopperschlager et al., 1977). For resolution of the question of the active form(s) of PFK by directly determining the enzymatically active aggregation state of the enzyme, active enzyme sedimentation was employed in this study. The results of such studies indicate that the predominant, if not the only, active form of PFK sediments with a  $s_{20,w}^0 = 12.4 \pm 0.5$  S at pH 8.55. No other detectable forms of active components were observed under the present experimental conditions and within the resolving power of such a technique. Although the sedimentation technique is powerful and the experimental generation of data is relatively straightforward, it has inherent limitations and pitfalls. Without realization of such problems, erroneous conclusions may be drawn from such measurements. Let us examine these limitations and their effects on the study of the active aggregation state of PFK.

A requirement for active enzyme sedimentation experiments is an adequate positive density gradient to stabilize the negative gradient associated with the leading edge of the sedimenting band. Without such a stabilizing gradient, the band of enzyme may "sink", leading to erroneous results. The stabilizing density gradient may be generated by the sedimentation of the small molecules in the substrate solution such as neutral salts, buffer, or  $D_2O$ . In the present study three solvent systems were employed, namely, 50% (v/v)  $D_2O$ , 10% (w/v) sucrose, and 4 or 10% (v/v) glycerol. The introduction of these solvents to protein solutions may lead to unexpected results if the interaction of protein and solvent is not considered, since (Cassassa & Eisenberg, 1964)

$$(\partial g_3 / \partial g_2)_{T, \mu_1, \mu_3} = \frac{(\partial \rho / \partial c_2)_{\mu_3} - (\partial \rho / \partial c_2)_{m_3}}{(\partial \rho / \partial c_3)_{m_2}} \quad (3)$$

where  $\partial g_3 / \partial g_2$  is the preferential interaction parameter between protein (component 2) and solvent (component 3).  $\partial \rho / \partial c_i = 1 - \phi_{i\rho}$  for component  $i$ , and the subscripts  $\mu_3$  and  $m_3$  indicate that the measurements are obtained at constant chemical potential and constant chemical composition, respectively. Substituting into eq 1 and rearranging

$$\frac{1}{s_{20,w}} = \frac{1}{s_{\text{obsd}}} \frac{\eta_{20,w}}{\eta_{t,w}} \frac{\eta_{t,w}}{\eta_{\text{solvent}}} \left[ 1 + \frac{1 - \bar{v}_3 \rho}{1 - \bar{v}_2 \rho} (\partial g_3 / \partial g_2) \right] \quad (4)$$

It is evident that, depending on the magnitude for  $\partial g_3 / \partial g_2$ , failure to consider such preferential interaction parameters may lead to significant differences in the value of sedimentation coefficients. Since  $\partial g_3 / \partial g_2$  is a measure of the amount

of solvent molecules (component 3) present in the immediate domain of the protein (component 2) over its concentration in the bulk solvent, it may assume either a positive or negative value. A positive value of this parameter indicates an excess of solvent molecules in the domain of the protein; a negative value means a deficiency of solvent molecules or an excess of water (component 1) in the immediate domain of the protein. A positive value of  $\partial g_3 / \partial g_2$  would lead to a lower corrected value for  $s_{20,w}$  than that if the preferential interaction was not taken into consideration. Conversely, a negative value of  $\partial g_3 / \partial g_2$  would yield a higher corrected value for  $s_{20,w}$ .

The results from measurements on preferential solvent interaction (Table III) indicate a negative value for  $\partial g_3 / \partial g_2$ , implying a preferential interaction of water with protein in both solvent systems. According to eq 4, the observed sedimentation coefficient without correcting for the preferential solvent parameter would assume a value lower than the corrected one. One may then erroneously conclude that the active form of PFK in these solvent systems assumes a smaller oligomeric state than that in  $D_2O$ . If the 12S form represents a tetrameric state of PFK, then these lower values of  $s_{20,w}$  may indicate the presence of active trimeric PFK. However, after more rigorous analysis by including preferential solvent interactions, the corrected values of  $s_{20,w}$  became  $12.4 \pm 0.3$ ,  $12.2 \pm 0.5$ , and  $12.8 \pm 0.7$  S in 4% (v/v) glycerol, 10% (v/v) glycerol, and 10% (w/v) sucrose, respectively. Such results are in good agreement with that observed in the presence of 50% (v/v)  $D_2O$ . The sedimentation coefficient of the active form of PFK in 4% (v/v) glycerol is in good agreement with that in 10% glycerol after correcting for preferential solvent interaction. Such an observation is another indication that the effects of the solvents on PFK are most likely nonspecific protein-solvent general thermodynamic interactions.

The presence of organic solvents not only induces preferential hydration of the protein but also apparently enhances the association constant of PFK. In all three solvent systems the apparent  $s_{20,w}$  values are higher than that at equivalent protein concentrations but without these solvent additives (Figure 6).  $D_2O$  is known to enhance the self-association of some protein systems (Timasheff, 1973; Pagliani & Lauffer, 1968; Lee & Berns, 1968; Baghurst et al., 1972), and the possible mechanism is that deuterium bonding is of greater strength than hydrogen bonding (Timasheff & Townend, 1969; Nemethy & Scheraga, 1964). The effects of glycerol and sucrose on PFK self-association are quite similar to those observed in microtubule assembly (Lee & Timasheff, 1977; Timasheff et al., 1976a). The proposed driving force is derived from a thermodynamically unfavorable interaction between protein and the solvent systems. It is, therefore, not surprising to observe an apparent enhancement of PFK self-association in the presence of these solvents. This leads to the following other questions. Can the observed 12S active form of PFK be the consequence of an artifact due to the presence of solvent additives? Is it possible that a smaller oligomeric form of PFK may also be active and the solvent additives enhance the self-association of these forms to the observed 12S state? It is unlikely that such is the case. The concentration of PFK employed ( $0.3\text{--}2 \mu\text{g/mL}$ ) in most of the active enzyme sedimentation experiments is much lower than  $10 \mu\text{g/mL}$ , the lowest concentration at which regular sedimentation velocity experiments were conducted. The extrapolated values for  $s_{20,w}$  at  $1\text{--}2 \mu\text{g/mL}$  would be much lower than 12 S even with solvent additives. Hence, the presence of these additives cannot solely account for the presence of the 12S component. The presence of substrates must have an effect on the association

of the smaller oligomeric form(s) to the 12S active component, a conclusion consistent with the reports by Hammes and co-workers. It may be concluded that the choice of solvent components as the stabilizing gradient is critical and the effects of these solvents on the protein system should be rigorously defined. Failure to consider such parameters may lead to erroneous conclusions.

A condition for the validity of the mathematical analysis in active enzyme sedimentation is the assumption that every enzyme molecule in the band reacts on its substrate with the same velocity. Deviation from such an assumption may occur if PFK undergoes denaturation during the course of the experiment and/or a depletion of substrate caused by insufficient substrate present initially or overloading the system with PFK. Let us examine the experimental results to determine if these assumptions were valid in the present study. If during the centrifugation procedure the enzyme activity is constant, the activity of the band of PFK must be equal to the activity of an equivalent amount of enzyme measured by steady-state kinetics in an identical substrate solution used for the centrifugation experiments. Such expectation is fulfilled for PFK within experimental precision as indicated by results shown in Table II. Furthermore, a linear relation was established for the total amount of product formed as a function of time of sedimentation under the present experimental conditions. Evidently there is no loss of activity due to partial "sinking" of PFK nor is there denaturation of the enzyme in the course of these experiments.

Depletion of substrate can lead to dramatic deviation from the actual sedimentation coefficient of the active component. Cohen & Claverie (1975) demonstrated that with an initial concentration of substrate equivalent to the Michaelis constant,  $K_m$ , and with a high consumption of substrate by the passage of a band of enzyme, the observed  $s_{20,w}$  could be 10% higher than the actual value. However, with an initial substrate concentration of 10-fold of  $K_m$  and with over 50% consumption of substrate, the observed value for  $s_{20,w}$  may only be ~1–2% higher. In the present study substrate concentrations of 4–100-fold of  $K_m$  have been routinely employed, and there is no observable dependence of  $s_{20,w}$  on substrate concentration. A similar higher artifactual  $s_{20,w}$  may be observed due to the presence of an excessive amount of enzyme. If such were the case, a positive slope is expected for the plot of observed  $s_{20,w}$  against amount of enzyme. In the present study, however, within the range of concentration of PFK employed, there is no observable dependence of  $s_{20,w}$  on the amount of PFK. Thus, it may be concluded that the amount of substrate depleted as a consequence of the passage of the band of PFK is not significant enough to affect the conclusion of this study. The 12S oligomeric form observed is most likely the actual active form of PFK.

In active enzyme sedimentation experiments, if the enzymatic reaction is measured through a coupling enzyme system, additional conditions have to be fulfilled, namely, the concentrations of the coupling enzymes must be sufficient to transfer the intermediate products rapidly so that it is not the rate-limiting step in the system. An insufficient amount of coupling enzymes may lead to an erroneous, lower sedimentation value (Cohen & Mire, 1971). In the present study, doubling the concentrations of coupling enzymes does not lead to any observable change in the sedimentation value of PFK; thus, the intermediate products are apparently rapidly utilized. An additional condition is that the sedimentation coefficients of the coupling enzymes should be lower than that of the enzyme studied. All coupling enzymes employed have sedi-

mentation coefficients much lower than 12 S. Thus, the present experimental conditions apparently satisfy the additional requirements for coupling enzyme systems. These additional conditions, however, demonstrate the limitation of such a system. If oligomeric forms smaller than the 12S component are active, then the coupling enzyme system is not capable of demonstrating the presence of these forms. The pH-dependent dye-linked system was employed to overcome such limitations. In such a system PFK is the only protein present and the presence of multiple forms may then be detected. Yet it is less sensitive than the coupling enzyme system, enabling one to utilize a larger amount of PFK, and the upper limit is determined by the magnitude of change in pH as a consequence of liberation of proton during enzyme reaction. The low buffering capacity of the system will not be able to maintain the pH. In the present study the maximum change of pH observed is limited to 0.02 unit, thus minimizing the effect of perturbation of pH in the aggregation state of PFK. The fact that an identical active form of PFK was observed in both assay systems and that in both systems essentially all the enzymatic activities are accounted for indicates that at least the predominant active form of PFK sediments with a  $s_{20,w}$  of  $12.4 \pm 0.5$  S.

Apparently there is one detectable active form for PFK under the experimental conditions. However, can multiple active components be detected by active enzyme sedimentation? How may the data be presented to facilitate detection of multiple active forms? Cohen & Claverie (1975) and Llewellyn & Smith (1978) simulated sedimentation profiles for different combinations of multiple active forms, be they products of self-assembly or complexing between active and inactive forms. It was concluded that multiple active forms of an enzyme can be detected by this technique. Recent reports in the literature on the active forms of pyruvate carboxylase (Taylor et al., 1978) and transcarboxylase (Poto & Wood, 1977) demonstrated that indeed multiple active forms can be monitored. As a consequence of these theoretical and practical considerations, an attempt was made to analyze the data more rigorously in search for an enzymatically active form of PFK other than the predominant 12S component. Cohen & Claverie (1975) and Llewellyn & Smith (1978) demonstrated the advantages of expressing the data as difference curves. One of the assumptions in such treatment is that the diffusion phenomenon is neglected. To minimize the complication of diffusion in the estimation of the sedimentation value, the recommended minimum angular velocity for a 12S component is ~45 000 rpm. Since the experiments in this study were all performed at 60 000 rpm, it may be concluded that analysis of data by difference curves would reflect closely the actual sedimentation behavior of PFK. When the difference curves generated were examined, be it for the coupling enzyme or the pH-dependent dye-linked system, they all demonstrated Gaussian distribution with no consistent skewness nor indication of the presence of second component. If there were other active species, the difference curves may exhibit bimodality (Cohen & Claverie, 1975). It is interesting to note that the difference curves generated from data for the crude preparation of PFK exhibit Gaussian distribution also.

In conclusion, the results presented in this study on the enzymatic active form of rabbit muscle PFK have shown the presence of a single component with a sedimentation coefficient of  $12.4 \pm 0.5$  S. Thus, it constitutes the first direct demonstration of the 12S species of rabbit muscle PFK being active. Such a conclusion is in accord with models proposed based on indirect measurements that the tetrameric form of PFK

is active. Under the experimental conditions employed and within the limits of resolution of the technique, no active component smaller than 12 S was detected. It is conceivable, however, that at PFK concentrations higher than those employed in this study, larger oligomeric forms of PFK may be present and are active. This study, however, does not yield direct information on the identity of the inactive form(s) of PFK. The results presented do not enable any conclusion on the mode of metabolic regulation to be made. Investigation is now undertaken hoping to provide information on these questions.

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