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**ARTICLE** *in* BIOCHEMISTRY · MAY 1985

Impact Factor: 3.02 · DOI: 10.1021/bi00331a011 · Source: PubMed



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# Subunit Interaction of Rabbit Muscle Phosphofructokinase: Effects of Purification Procedures†

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ABSTRACT: Structural, physical, and kinetic properties of rabbit muscle phosphofructokinase (PFK) purified by three different procedures were monitored in order to determine the effect of various purification procedures on the dynamics of subunit interaction. PFK was purified by three commonly used procedures: (1) differential heat precipitation [Kemp, R. G. (1972) *Methods Enzymol. 42*, 71–77], (2) differential heat and alcohol precipitation [Ling, R. H., Marcus, F., & Lardy, H. A. (1965) *J. Biol. Chem. 240*, 1893–1899], and (3) differential salt fractionation [Hesterberg, L. K., & Lee, J. C. (1980) *Biochemistry 19*, 2030–2039]. The physical, kinetic, and structural properties of these three preparations show that these proteins are not identical. Sedimentation velocity studies show that PFK purified by method 3 self-associates rapidly and that the system is thermodynamically homogeneous. The presence of an inactive or noninteracting component is not observed within an 8-h time limit. In contrast, PFK purified by method 1 or 2 is heterogeneous. In these preparations, a slowly sedimenting, noninteracting, inactive form of PFK is present. The remaining active protein is not stable but continuously converts to an inactive form. Active PFK can be fractionated from this inactive form by sedimentation. This active fraction behaves as a thermodynamically homogeneous system, and the subunits undergo rapid association–dissociation in a manner similar to PFK purified by method 3. Kinetic studies on these three preparations show that the inclusion of a heat and/or alcohol step in the purification procedure yields an enzyme that is less stable, has a lower specific activity, requires DTT for full activation, and is more susceptible to inhibition by ATP. PFK purified by method 2 has been demonstrated to have a lower subunit molecular weight by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and peptide mapping. This study demonstrates unequivocally the detrimental effects of including heat and alcohol precipitation steps in the purification procedure for PFK. It also shows that the various oligomeric states of *native* PFK subunits are in a dynamic, rapid equilibrium and that the kinetic parameters of active PFK are dependent on protein concentration; thus, these results demonstrate that the self-assembly of PFK subunits must play a role in the regulation of PFK activity.

Rabbit muscle phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) (PFK)[1] is a key glycolytic enzyme which is allosterically regulated. Steady-state kinetic studies have led to the proposal of a model for the regulation of PFK. This model states that the enzyme exists in two conformational states, namely, an active (R) and inactive (T) form, and that these two forms are assumed to represent conformational isomers of the tetrameric enzyme (Goldhammer & Hammes, 1978; Roberts & Kellet, 1979, 1980a,b; Frieden et al., 1976). Frieden and co-workers propose that the inactivation or activation of the enzyme depends on the protonation or deprotonation of certain ionizable groups on this tetramer (Bock & Frieden, 1976a,b; Frieden et al., 1976; Pettigrew & Frieden, 1979a,b). Ligands perturb the equilibrium by preferentially binding to either the protonated (inactive) or unprotonted (active) forms of PFK and concomitantly shifting the apparent p$K$ of important ionizable groups. In this proposed model, subunit association–dissociation plays no role due to the apparently slow rate constants governing this interaction (Bock & Frieden, 1976a,b; Frieden et al., 1976; Pettigrew & Frieden, 1979a,b), although the aggregation state of PFK has been shown to be influenced by the presence of various metabolities (Parmeggiani et al., 1966; Lad et al., 1973; Aaronson & Frieden, 1972; Hill & Hammes, 1975; Hesterberg & Lee, 1982, Luther et al., 1983).

Recently, studies from this laboratory have established that PFK is capable of undergoing association–dissociation in a rapid, dynamic equilibrium when studied by sedimentation velocity (Hesterberg & Lee, 1981, 1982; Luther et al., 1983). These studies have shown that the simplest mode of association can best be described as $M_1 \rightleftharpoons M_2 \rightleftharpoons M_4 \rightleftharpoons M_{16}$. As clearly demonstrated, this self-association is perturbed by ligands. In general, activators favor the formation of larger aggregates whereas inhibitors favor that of the smaller aggregates, in particular, that of the dimer (Hesterberg & Lee, 1982). Furthermore, direct evidence has been presented to demonstrate the presence of two tetrameric forms of PFK that differ in their hydrodynamic properties (Hesterberg & Lee, 1980; Hesterberg et al., 1981; Luther et al., 1983). Active tetramer sediments as a 12.4S particle, while inactive tetramer exists as a 13.5S molecule. Since the self-association of PFK is in equilibrium with these two conformational states, then thermodynamically the association–dissociation of PFK subunits must play a role in the regulation of PFK activity (Luther et al., 1983). One of the issues on the mechansm of regulation of rabbit muscle PFK is the mode of subunit association, i.e., whether it is rapid or slow as studied by sedimentation velocity.

One of the obvious factors that is different among these reports is in the procedures for purifying the enzyme. An outstanding feature is the omission or inclusion of differential heat denaturation and/or alcohol precipitation steps which

† This work was supported by National Institutes of Health Grants NS-14269 and AM-21489.

[1] Abbreviations: PFK, phosphofructokinase; F6P, fructose 6-phosphate; SDS, sodium dodecyl sulfate; TEMA buffer, 75 mM Tris–$CO_3$ with 3 mM EDTA, 18 mM $MgCl_2$, and 9.0 mM $(NH_4)_2SO_4$ buffer at pH 7.0; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

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desensitize some allosteric enzymes (Kurganov, 1982; Stadtman, 1966; Seya & Nagasawa, 1981). It is conceivable that PFK samples demonstrate different behavior as a consequence of different degrees of desensitization occurring during purification. The major goal of this investigation, therefore, is to thoroughly compare the kinetic, chemical, and physical properties of PFK purified by these various procedures so as to resolve the issue of whether native PFK undergoes rapid association–dissociation.

## Materials and Methods

ATP, F6P, DTT, iodoacetamide, CNBr, and β-mercaptoethanol were purchased from Sigma Chemical Co. Aldolase, glycerol-3-phosphate dehydrogenase–triosephosphate isomerase, pyruvate kinase, lactate dehydrogenase, fructose-1,6-bisphosphatase, and NADH were obtained from Boehringer-Mannheim. Ultrapure grade acrylamide was supplied by Bethesda Research Laboratories, Inc. Sodium dodecyl sulfate (SDS) and ultrapure grade urea were obtained from Bio-Rad and Schwarz/Mann, respectively. These were all used without further purification.

PFK samples used in these studies were purified by three different methods—Ling et al. (1965), Kemp (1972), and Hesterberg & Lee (1981). PFK purified by these three methods will be referred to as L-PFK, K-PFK, and H-PFK, respectively. For the three purification procedures, all steps were conducted at 4 °C.

The purification procedure of Hesterberg & Lee (1980) has only been briefly outlined before; therfore, it will be presented in more detail. Purification of PFK by this method follows the procedure of Hussey et al. (1977) with slight modifications.

A total of 500–800 g of fresh rabbit muscle was ground and homogenized in a Waring blender for two periods of 30 s each in 3 volumes (w/v) of 10 mM phosphate, 30 mM KF, and 3 mM EDTA at pH 7.5. The homogenate was centrifuged at 10000*g* for 50 min. The pellet was discarded, and the pH of the supernatant was raised to 7.5 with the addition of 4 M $NH_4OH$. Ammonium sulfate was then added slowly to the supernatant with stirring until 45% saturation was attained. This suspension was stirred for 1 h and then centrifuged at 10000*g* for 50 min. The resulting supernatant was retained, and additional ammonium sulfate was added, as before, up to 55% saturation. The solution was stirred for 1 h and allowed to stand undisturbed for at least 8 h. The precipitate, containing PFK, was collected by centrifuging at 10000*g* for 50 min, and then gently dissolved in a minimum volume of 50 mM Tris–phosphate, 1 mM EDTA, 1 mM DTT, 0.2 mM fructose 1,6-bisphosphate at pH 8.0 (buffer A). The PFK solution was applied to a 40 × 500 mm column packed with Bio-Gel A-1.5m and preequilibrated with 50 mM Tris–phosphate, 1 mM EDTA, 1 mM DTT, 0.2 mM fructose 1,6-bisphosphate, and 1 M $(NH_4)_2SO_4$ at pH 8.0 (buffer B). The column was eluted with buffer B. Fractions were assayed for PFK activity, and those fractions with a specific activity of ≥80 units/mg were combined. Ammonium sulfate was added with stirring to this solution up to 55% saturation. After being stirred for 1 h, the solution was allowed to stand undisturbed for at least 8 h. The precipitate was collected by centrifuging at 10000*g* for 50 min. The resulting pellet was gently dissolved in a minimum volume of buffer A. The PFK solution was layered onto a 22 × 600 mm column packed with Bio-Gel A-1.5m that was preequilibrated with buffer A. The column was eluted with buffer A, and those fractions with a PFK specific activity ≥150 units/mg were combined. Ammonium sulfate was added to this solution with stirring until 60% saturation was obtained. The solution was then stirred for 1 h and allowed to sit at least 8 h. The resulting precipitate was collected by centrifuging at 10000*g* for 50 min and resuspended in a minimum volume of 50 mM Tris–phosphate and 0.1 mM EDTA at pH 8.0 (buffer C). The solution was dialyzed for at least 8 h against 4 L of buffer C. The dialyzed sample was then applied to a 40 × 200 mm column packed with DEAE-Sephadex A-50 and preequilibrated with buffer C. The column was washed with 500 mL of buffer C, and the PFK was then eluted with 500 mL of 0.3 M Tris–phosphate and 0.1 mM EDTA at pH 8.0. Those fractions with a PFK specific activity of ≥300 units/mg were pooled. Ammonium sulfate was added to the PFK solution up to 80% saturation. After being stirred for 1 h, the solution was allowed to stand for at least 6 h. The precipitate was collected by centrifuging at 10000*g* for 50 min. The resulting pellet was gently resuspended in a solution of buffer C saturated with ammonium sulfate and stored at 4 °C.

In all experiments PFK was equilibrated in the appropriate buffer by passage through a Sephadex G-25 column (1.2 × 8.5 cm). The concentration of enzyme was determined by the absorbance at 280 nm using an absorptivity coefficient of 1.07 L/(g·cm) (Hesterberg & Lee, 1981). PFK from all three sources was assayed for activity and regulatory properties as previously described (Hesterberg & Lee, 1981). H-PFK activity was further analyzed as a function of protein concentration by three different procedures—two coupled enzyme and a pH-stat assay. The first coupled enzyme procedure involved aldolase, glycerol-3-phosphate dehydrogenase–triosephosphate isomerase as described by Hesterberg & Lee (1981). The second coupled enzyme procedure employed pyruvate kinase, lactate dehydrogenase, and fructose-1,6-bisphosphatase in TEMA buffer as described by Emerk & Frieden (1975). The third enzyme assay procedure was a modified pH-stat procedure of Dyson & Noltman (1965). The standard reaction mixture in the latter procedure contained 1 mM ATP, 5 mM $MgCl_2$, 10 mM KCl, and 65 μM glycylglycine at pH 7.0. PFK was equilibrated in a buffer consisting of 12.5 mM glycylglycine and 1 mM EDTA at pH 7.0. Base consumption as a function of time was controlled and recorded on a Radiometer titration system with 1 mM NaOH as titrant.

Carboxamidomethylated derivatives of L-PFK, K-PFK, and H-PFK were prepared according to the procedure of George et al. (1981).

SDS–polyacrylamide slab gel electrophoresis was carried out according to the procedure of Laemmli (1970). Gels of 4, 6, 8, 10, and 12% (w/v) acrylamide were used instead of an acrylamide gradient for apparent molecular weight determinations. PFK from the three preparations were analyzed for molecular weight differences by the procedure of Hedrick & Smith (1968). Gels were stained by Coomassie Brilliant Blue or ammonical silver as described by Field et al. (1984).

Peptide mapping studies on the three PFK samples were performed according to a modified procedure of Hashimoto et al. (1983) as described by Field et al. (1984).

All densitometric scannings of the gels for molecular weight determinations and peptide maps were performed on an LKB-2202 Ultroscan laser densitometer equipped with an LKB 2200 recording integrator.

Two specific sedimentation experiments were conducted to test for dynamic equilibrium in PFK subunit interaction. First, the relation between weight average sedimentation coefficient and PFK concentration was tested as a function of angular velocity. Second, the reaction boundary obtained at a high PFK concentration was fractionated into a leading and trailing

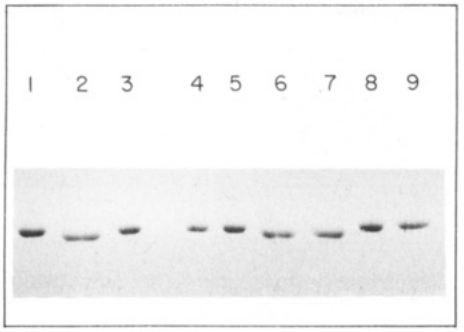


FIGURE 1: SDS–PAGE of PFK preparations. The trailing fraction of L- and K-PFK was refractionated as described under Materials and Methods. The slow-moving component was isolated and analyzed along with the corresponding unfractionated and leading fraction forms of the PFK preparations. The samples in each lane are the following: lane 1, unfractionated H-PFK; lane 2, unfractionated L-PFK; lane 3, unfractionated K-PFK; lane 4, trailing fraction H-PFK; lane 5, leading fraction H-PFK; lane 6, slow-moving component L-PFK; lane 7, leading fraction L-PFK; lane 8, slow-moving component K-PFK; lane 9, leading fraction K-PFK.

fraction by sedimentation velocity experiments employing an aluminum partition centerpiece. A 0.8-mL sample of a stock solution of PFK at 5 mg/mL in buffer was placed in an ultracentrifuge cell containing an aluminum partition centerpiece and was centrifuged at 60 000 rpm in an An-D rotor at 23 °C. This was continued until at least 80% of the reaction boundary had passed through the partition as monitored by schlieren optics. The fraction passing through the partition was referred to as the leading fraction, while that which did not was the trailing fraction. At the completion of centrifugation, the leading and trailing fractions were carefully removed. The protein concentration was then determined for each of these fractions. PFK from these two fractions as well as the unfractionated form were all diluted to the same protein concentration. In order to directly monitor these fractionated and unfractionated samples for rapid association–dissociation behavior, sedimentation velocity studies were conducted to monitor both the sedimentation patterns and $\bar{s}_{20,w}$, the weight average sedimentation coefficient.

Further fractionation of the trailing fraction of L-PFK and K-PFK was performed because initial separation using the partition cell revealed two components in this fraction even at low PFK concentrations. The trailing fraction was isolated as described above and diluted to 1.0 mg/mL. This sample was then fractionated once more by sedimentation.

Sedimentation velocity experiments were analyzed by previously described procedures (Hesterberg & Lee, 1981). Values for $\bar{s}_{20,w}$ were determined from the centroid, a close approximation of the second moment, of the scanner-traced boundaries. The observed weight average coefficients were normalized to standard conditions by correcting for solvent viscosity and density. All experiments were conducted in 75 mM Tris–carbonate, 18 mM $MgCl_2$, 9 mM $(NH_4)_2SO_4$, and 3 mM EDTA (TEMA) at pH 7.0, 23 °C. For sedimentation velocity experiments, sapphire windows with Kel-F-coated aluminum double-sector centerpieces were used, except in the fractionation studies. The apparent partial specific volume of PFK in native conditions for H-PFK is 0.730 (Hesterberg & Lee, 1982) and was assumed to be the same for L-PFK and K-PFK, also.

## RESULTS

The purified PFK samples when analyzed by SDS–PAGE are all about 95% homogeneous, as shown in lanes 1–3 of Figure 1. However, the band corresponding to L-PFK migrates faster than that of H-PFK and K-PFK, suggesting that the subunit molecular weight of L-PFK is smaller. This observed difference may also be caused by factors other than a decrease in molecular weight such as differential interaction between protein and SDS or incomplete unfolding of proteins. In order to resolve this observed difference in mobility, the PFK samples were all further tested by SDS–PAGE as a function of acrylamide concentration, and the results were analyzed according to the procedure of Hedrick & Smith (1968). The slope of the $R_f$ vs. percent acrylamide plot for L-PFK is lower than that of H-PFK and K-PFK, but the $y$ intercepts for all these plots are identical, indicating that this difference between these PFK samples is only in molecular weight. L-PFK has an apparent molecular weight approximately 7000 (±3000) less than H-PFK and K-PFK. The observed values for L-PFK, K-PFK, and H-PFK are $M_r$ 75 000, 81 000, and 83 000, respectively. These values are all within the range of the molecular weights previously reported in the literature for PFK (Hesterberg et al., 1982; Uyeda, 1969; Leonard & Walker, 1972; Pavelich & Hammes, 1973).

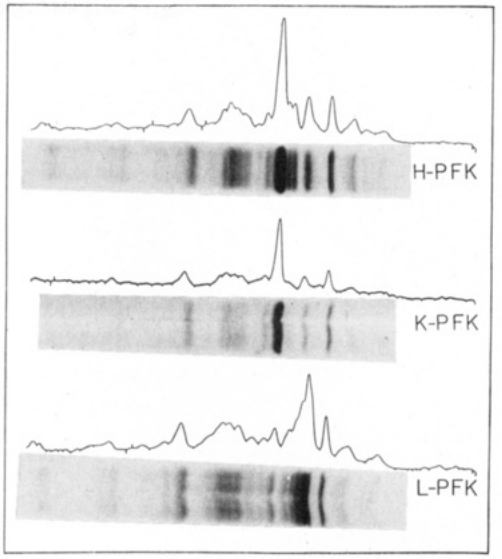


FIGURE 2: One-dimensional peptide mapping of PFK preparations. PFK was exhaustively digested with CNBr, and the resulting peptides were separated by SDS–PAGE and silver stained as described under Material and Methods. The corresponding densitometric scans of the gel are shown above the gel.

In order to compare the chemical properties of PFK subunits at a higher level of resolution, carboxamidomethylated derivatives of PFK samples from these preparations were cleaved exhaustively with CNBr. The identity of the peptides generated was studied by electrophoresis on a 10–18% acrylamide gradient SDS–PAGE. Results of this one-dimensional peptide map are shown in Figure 2. The number and identity of these peptides are essentially identical between the H-PFK and K-PFK samples. The relative intensity of these peptides is also similar. Hence, it can be concluded that there is no detectable chemical difference between H-PFK and K-PFK. In the case of L-PFK a very significant difference can be detected. The most prominent peptide that is present in H-PFK and K-PFK is absent in L-PFK. Instead, an equally prominent peptide with a lower apparent molecular weight can be detected in L-PFK. The observation of a smaller subunit molecular weight for L-PFK suggests that this smaller peptide in L-PFK is a consequence of proteolytic cleavage of the larger peptide in K- and H-PFK.

Having established the basic chemical properties of these PFK samples, the steady-state kinetic behavior of these sam-

Table I: Kinetic Parameters of Different PFK Preparations[a]

| preparation | $K_m$ ($\mu$M) of ATP | $K_m$ ($\mu$M) of ITP | $K_m$ ($\mu$M) of F6P | $K_I$ (mM) of ATP | $n^b$ | specific activity (units/mg) +1 mM DTT | specific activity (units/mg) −1 mM DTT |
|---|---|---|---|---|---|---|---|
| H-PFK | 50 ± 5 | 76 ± 5 | 100 ± 5 | 5.0 ± 0.1 | 1.9 ± 0.1 | 310 | 315 |
| K-PFK | 48 | 84 | 94 | 4.0 | 1.8 | 241 | 163 |
| L-PFK | 50 | 153 | 96 | 1.6 | 3.2 | 159 | 71 |

[a] All experiments were performed in TEMA buffer at pH 7.0, 23 °C. [b] $n$ is the Hill coefficient for F6P as the variable substrate, and a unit is defined as 1 $\mu$mol of substrate converted to product per minute.

ples was investigated. The effect of DTT on the enzymatic activity was tested. Results in Table I show that maximum activity is observed only if 1 mM DTT was added to L-PFK and K-PFK, yet H-PFK showed no increase in activity upon addition of DTT. However, even in 1 mM DTT the specific activities of L-PFK and K-PFK are not as high as that of H-PFK which has a specific activity 1.5- and 2-fold greater than that of K-PFK and L-PFK, respectively, as shown in Table I.

A more quantitative assessment of the kinetic properties for these PFK samples was conducted by monitoring the Michaelis constants, $K_m$, for the substrates F6P and ATP and the substrate analogue ITP at pH 7.0. Analysis of the kinetic data according to Lineweaver & Burk (1934) reveals that the $K_m$ values for the two substrates, F6P and ATP, are the same for all three preparations. However, the $K_m$ value for ITP is higher in L-PFK than that for both H-PFK and K-PFK, indicating a decrease in affinity of ITP for L-PFK. Further analysis of the F6P kinetic data by Hill plots (Hill, 1910) for each preparation shows that cooperativity is much greater in L-PFK than in K-PFK or H-PFK which are approximately the same. Results are summarized in Table I.

Since ATP is both a substrate and an inhibitor of PFK, inhibition studies were conducted to determine the dissociation constant, $K_I$, of ATP for all three preparations using the relation:

$$\frac{1}{v} = \frac{K_m}{V_{max}}\frac{1}{[s]} + \frac{1}{V_{max}} + \frac{[s]}{V_{max}K_I} \quad (1)$$

Table I reveals that the $K_I$ of ATP for H-PFK is higher than that of L-PFK and K-PFK with the $K_I$ of ATP for L-PFK being lower than that for K-PFK. This indicates that L-PFK and K-PFK are more sensitive to ATP inhibition than H-PFK.

The results of these kinetic regulatory studies show that the three preparations of PFK have similar properties; however, differences do exist. These differences, though, cannot provide direct information on the role of self-association in the regulation of PFK. Therefore, the self-association properties of the three preparations were investigated.

In an effort to establish whether the three PFK preparations undergo rapid self-association, the following tests were conducted. First, the $\bar{s}_{20,w}$ of the three PFK preparations were determined at 40 000 and 60 000 rpm. If a self-associating system were in slow equilibrium, the values of $\bar{s}_{20,w}$ would then be expected to change as a function of the angular velocity, $\omega^2$. Results in Table II show that H-PFK has identical $\bar{s}_{20,w}$ values at both speeds. Therefore, it may be concluded under these conditions H-PFK undergoes rapid self-association. However, for L-PFK and K-PFK a change in $\bar{s}_{20,w}$ is observed, which indicates the presence of a noninteracting component or a system characterized by a slow equilibrium.

The second test involves diluting a stock solution of PFK at 5.0 mg/mL to 300 $\mu$g/mL at time zero. Aliquots of the diluted PFK samples from each of the preparations were removed and analyzed by sedimentation as a fraction of time from 0 to 4 h after initial dilution. If the system were in rapid

Table II: Tests for Rapid Self-Association for PFK Preparations[a]

| | $\bar{s}_{20,w}$ for preparation H-PFK | $\bar{s}_{20,w}$ for preparation K-PFK | $\bar{s}_{20,w}$ for preparation L-PFK |
|---|---|---|---|
| speed (rpm) | | | |
| 40 000 | 13.9 ± 0.2 | 13.3 ± 0.2 | 13.1 ± 0.2 |
| 60 000 | 13.8 | 12.4 | 11.6 |
| time after dilution (h)[b] | | | |
| 0 | 13.9 ± 0.2 | 13.3 | 13.1 |
| 2 | 13.8 | 12.4 | 11.5 |
| 4 | 13.8 | 11.4 | 11.0 |

[a] All experiments were performed in TEMA buffer, at pH 7.0, 23 °C. The protein concentration for each of the preparations was 300 $\mu$g/mL. [b] At 60 000 rpm.

equilibrium, the value of $\bar{s}_{20,w}$ at zero time should be characteristic of the lower protein concentration and should not change as a function of time. Results show that $\bar{s}_{20,w}$ for H-PFK remains constant throughout the experiment of 4 h, indicating that a rapid equilibrium exists under the experimental conditions, as shown in Table II. K-PFK and L-PFK, however, show a decrease in $\bar{s}_{20,w}$ over a period of 4 h, indicating the presence of a slow equilibrium or the presence of noninteracting components.

The third test involves the fractionation of PFK samples and testing the sedimentation behavior of these fractions. At 5 mg/mL under the experimental conditions described, PFK exhibits a trimodal sedimentation profile. PFK samples from the three preparations were further tested for rapid self-association by separating the trimodal sedimentation pattern into two fractions using an aluminum partition centerpiece. The leading fraction is the one that has passed the partition whereas the trailing fraction is the one that has not. The two fractions were isolated and diluted to the same protein concentration as also was the unfractionated PFK for each of these preparations. The three samples were then subjected to sedimentation velocity studies. Figure 3A reveals that the leading, trailing, and unfractionated samples of H-PFK all show similar sedimentation patterns with no indications of bimodality. The observed values for $\bar{s}_{20,w}$ and specific activities are essentially identical. These results are indicative of a system undergoing rapid association–dissociation, thus confirming previously published results (Hesterberg & Lee, 1981, 1982; Luther et al., 1984). However, identical experiments yield different results using K-PFK and L-PFK. Figure 3B reveals that the leading and unfractionated forms of K-PFK have similar sedimentation patterns. They show no bimodality with $\bar{s}_{20,w}$ values of 14.6 and 14.2 S, respectively. However, the trailing fraction has a different sedimentation pattern with a lower $\bar{s}_{20,w}$ value of 11.0 S. Upon closer examination this trailing fraction is seen to be composed of two components; a fast-moving component and a slow-moving component with $\bar{s}_{20,w}$ of 13.5 and 4.88 S, respectively. The specific activities of these three fractions are also different for K-PFK. The leading fraction has a higher specific activity than the unfractionated form, while the trailing fraction has an even lower specific activity. Further separation of the slow-moving component from the fast component in this trailing fraction reveals that this

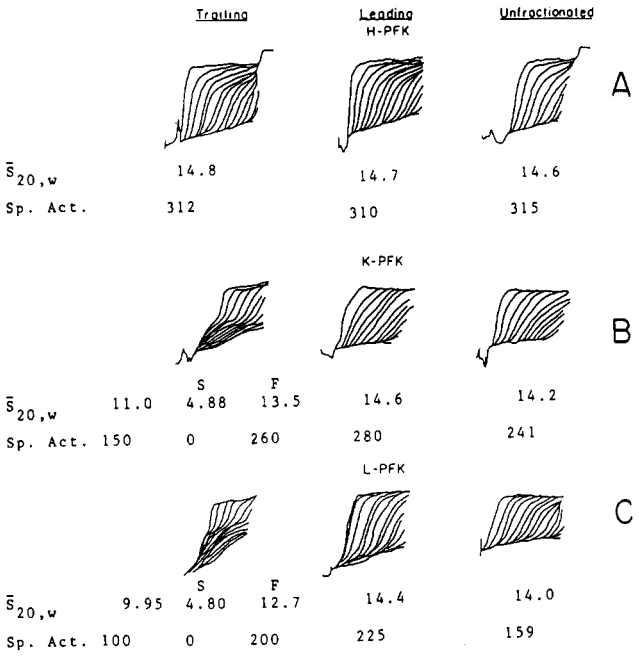


FIGURE 3: Summary of the sedimentation and kinetic properties of PFK fractions in TEMA buffer at pH 7.0, 23 °C. (A) H-PFK, (B) K-PFK, and (C) L-PFK. PFK concentration for each preparation and fraction was 400 μg/mL. S and F represent the slow- and fast-moving component, respectively.

slow-moving component possesses no PFK activity while the fast-moving component has a specific activity of 260 which is similar to that of the leading fraction of 280, as shown in Figure 3B. Similar results were also obtained for the L-PFK as shown in Figure 3C. The results for K-PFK and L-PFK indicate that these samples contain a component(s) that is not in rapid equilibrium with active PFK.

Since H-PFK is the only preparation that apparently can be considered to undergo rapid equilibrium, it is further subjected to a test for reversibility. Protein samples at 25 μg/mL were prepared. An aliquot of the solution was subjected to sedimentation velocity measurement, and another aliquot was concentrated and subsequently subjected to sedimentation velocity measurement also. Results from seven experiments showed that at 25 μg/mL H-PFK sediments with an $\bar{s}_{20,w}$ of 9.1 ± 0.2 S and the reconcentrated samples of 100 μg/mL sediment with an $\bar{s}_{20,w}$ of 12.3 ± 0.3 S. These values are in exact agreement with H-PFK samples diluted from a stock solution of 5 mg/mL. Hence, it can be concluded that the association–dissociation of H-PFK is reversible.

To further analyze that the component present in the L-PFK and K-PFK trailing fractions, this component was isolated and subjected to SDS–PAGE analysis. Figure 4 reveals that the slow-moving component of both L-PFK and K-PFK comigrates with its respective unfractionated and leading fraction form. This indicates that the slow-moving component is either PFK or a contaminant of equal apparent molecular weight. If the slow-moving component arises as a result of a slow conversion of PFK from one form to another, the formation of this component should be observable as a function of time. Initially upon fractionation, the leading fractions of L-PFK and K-PFK show no indication of any slow-moving component, as shown in Figure 3B,C. Therefore, the leading fractions were employed as the *starting material* to monitor the formation of this slow component by simultaneously measuring both the sedimentation and specific activity as a function of time. Sedimentation results demonstrate that the appearance of the slow-moving component is time dependent with more than 30%

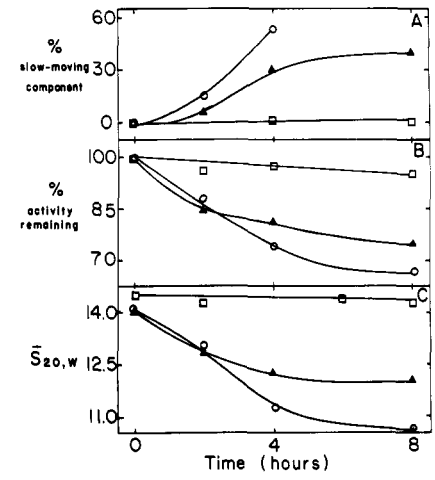


FIGURE 4: Protein stability of the leading fraction of PFK preparations as a function of time. Symbols and preparations are as described in Figure 1. The leading fractions were isolated as described under Material and Methods. Values for specific activities and the amount slow-moving component are normalized. (A) Percent slow-moving component vs. time. Amount of slow-moving component was determined by calculating what percent of absorbance at 280 nm the slow-moving component contributed to the trailing fraction. (B) Percent activity remaining vs. time. One-hundred percent activity corresponds to 310 units/mg for H-PFK, 240 units/mg for K-PFK, and 160 units/mg for L-PFK. (C) $\bar{s}_{20,w}$ vs. time. PFK concentration for each preparation was 400 μg/mL. All experiments were conducted in TEMA buffer, pH 7.0 at 23 °C.

Table III: Kinetic Parameters of the Leading Fractions for PFK Preparations[a]

| preparation | $K_m$ (μM) of ATP | ITP | F6P | $K_I$ (mM) of ATP | $n^b$ |
|---|---|---|---|---|---|
| H-PFK | 48 ± 5 | 74 ± 5 | 90 ± 5 | 4.8 ± 0.1 | 1.8 ± 0.1 |
| K-PFK | 47 | 81 | 90 | 4.5 | 1.6 |
| L-PFK | 55 | 151 | 94 | 4.0 | 2.0 |

[a] All experiments were performed in TEMA buffer at pH 7.0, 23 °C, on protein samples that were used within 1 h of preparation. [b] $n$ is the Hill coefficient for F6P as the variable substrate.

of the L-PFK and K-PFK sedimenting as the slow-moving component by the end of the test period of 8 h. In marked contrast, the H-PFK sample did not show any detectable slow-moving component under these experimental conditions, as shown in Figure 4A. The appearance of the slow-moving component coincided with a decrease both in specific activity and in $\bar{s}_{20,w}$, as shown in Figure 4B,C. These results indicate that this slow-moving, inactive PFK seen initially in the trailing fraction of L-PFK and K-PFK arises as the result of denaturation or irreversible conversion of L-PFK and K-PFK from an active, native form to an inactive, slow-moving one.

The kinetic and sedimentation properties of L-PFK and K-PFK in the absence of this slow-moving component were studied. These studies were confined to the leading fractions of L-PFK and K-PFK which were prepared freshly and used within 1 h after fractionation. Analysis of the kinetic data reveals that the $K_m$ values for F6P, ATP, and ITP for the leading fractions of L-PFK and K-PFK are similar to those of their respective unfractionated forms, as summarized in Table III. Differences, however, are seen in the $K_I$ for ATP. The leading fractions of L-PFK and K-PFK exhibit a $K_I$ of 4.0 and 4.5 mM, respectively. These values are higher than the corresponding values of 1.6 and 4.0 mM determined for the unfractionated samples. Further analysis of the F6P kinetic data by Hill plots reveals that the leading fractions of L-PFK and K-PFK have lower Hill coefficients than their

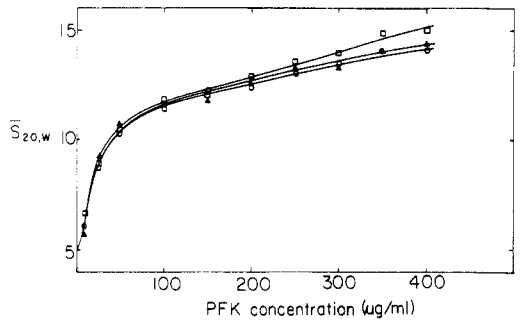


FIGURE 5: $\bar{s}_{20,w}$ as a function of PFK concentration for the PFK preparations in TEMA buffer at pH 7.0, 23 °C. The symbols and preparations of PFK are as described in Figure 1. The lines represent the theoretical fit of the experimental data using the association model of $M_1 \rightleftharpoons M_4 \rightleftharpoons M_{16}$ and other parameters shown in Table IV. The data points are the average of multiple data sets.

Table IV: Self-Association Studies on Active PFK in TEMA Buffer at pH 7.0, 23 °C

| preparation | $s_4^0$ (S) | stoichiometry | $K_4^{app}$ [(mL/mg)$^3$] | $K_{16}^{app}$ [(mL/mg)$^{15}$] | $\sigma$ |
|---|---|---|---|---|---|
| H-PFK | 13.5 | 1:4:16 | $5.00 \times 10^5$ | $2.5 \times 10^{23}$ | 0.16 |
| K-PFK | 13.5 | 1:4:16 | $4.08 \times 10^5$ | $7.18 \times 10^{22}$ | 0.19 |
| L-PFK | 13.2[a] | 1:4:16 | $3.44 \times 10^5$ | $5.00 \times 10^{22}$ | 0.05 |

[a] This is determined independently on an oxidized glutathione-inactivated sample in the presence of F6P and AMP-PNP by using a previously published procedure (Luther et al., 1983).

respective unfractionated forms. Similar studies on the leading fraction of H-PFK reveal no differences between the leading fraction and unfractionated forms, as summarized in Table III. Evidently, in the absence of the slow-moving, inactive component L-PFK and K-PFK appear to behave kinetically more like H-PFK.

In order to determine if in the absence of the slow-moving component L-PFK and K-PFK undergo rapid association–dissociation, freshly prepared leading fractions of L-PFK and K-PFK were further subjected to sedimentation studies. Two criteria previously used were applied to test if PFK in these fractions undergoes rapid, dynamic association–dissociation. These criteria were the measurement of $\bar{s}_{20,w}$ as a function of angular velocity and dependence of the value for $\bar{s}_{20,w}$ as a function of time of dilution from a stock solution of higher protein concentration. Sedimentation results show that the values of $\bar{s}_{20,w}$ are not dependent on angular velocity or time of dilution. Hence, it may be concluded that PFK samples in the absence of slow-moving inactive PFK components can undergo rapid, dynamic association–dissociation. Additional sedimentation velocity experiments were conducted as a function of enzyme concentration over the range of 10–500 $\mu$g/mL of freshly prepared leading fractions. Figure 5 shows the relation between $\bar{s}_{20,w}$ and PFK concentration. It is evident that the leading fractions of L-PFK and K-PFK self-associate in a manner similar to that of H-PFK. Results from these sedimentation velocity experiments were further analyzed by using previously published procedures (Hesterberg & Lee, 1981). The parameters employed in generating the theoretical curves are shown in Figure 5 and are summarized in Table IV. The simplest model which best fits the data for L-PFK, K-PFK, and H-PFK yields a stoichiometry of $M_1 \rightleftharpoons M_4 \rightleftharpoons M_{16}$ where $M_4$ is a tetramer assuming a sedimentation coefficient of 13.5 S for K-PFK and H-PFK, whereas the value for $s_4^\circ$ is 13.2 S for L-PFK. The apparent equilibrium constants $K_4^{app}$ for H-PFK, K-PFK, and L-PFK are similar and are in good agreement with previously published results (Hesterberg & Lee, 1982; Luther et al., 1983). These results imply that native L-PFK and K-PFK undergo rapid self-association in a manner similar to H-PFK.

Table V: Effect of PFK Concentration on Steady-State Kinetic Parameters[a]

| method of assay | protein concn ($\mu$g/mL) | $K_m$ ($\mu$M) | $n$ | $K_I$ (mM) |
|---|---|---|---|---|
| A | 0.1 | 92 ± 5 | 1.9 ± 0.1 | 5.0 ± 0.1 |
| | 0.8 | 128 ± 8 | 1.2 ± 0.1 | 6.0 ± 0.1 |
| B | 0.1 | 93 ± 6 | 1.4 ± 0.1 | |
| | 0.8 | 114 ± 7 | 1.1 ± 0.1 | |
| C | 0.6 | 700 ± 20 | 1.40 ± 0.1 | |
| | 1.0 | 1030 ± 40 | 1.1 ± 0.1 | |

[a] Methods A and B were the coupled enzyme procedures employing aldolase–glycerol-3-phosphate dehydrogenase–trisosephosphate isomerase and pyruvate kinase–lactate dehydrogenase–fructose-1,6-biphosphatase, respectively. Method C was the pH-stat procedure. $K_m$ and $K_I$ were obtained for F6P and ATP, respectively. $n$ was the Hill coefficient for F6P serving as the variable substrate. The significantly higher values for $K_m$ observed in using method C are due to the much lower ionic strength buffer employed as dictated by the intrinsic nature of the method.

In this current working hypothesis, subunit self-assembly of PFK plays a role in the regulation of enzyme activity; hence, it is of interest to monitor enzyme activity as a function of protein concentration. Different coupling enzymes were employed to overcome artifacts that may be associated with the various assay procedures as discussed by Emerk & Frieden (1975). Results summarized in Table V show that steady-state kinetic parameters of PFK are dependent on protein concentration regardless of the method of assay. Within the limited scope of this study, an increase in the value of $K_{m,F6P}$ is seen to be associated with an increase in protein concentration. Concomitantly, a decrease in Hill coefficient is also observed which suggests a lowering of the degree of cooperativity with increasing protein concentration. In addition, at a higher protein concentration the value of $K_{I,ATP}$ is higher; thus, it indicates that PFK is less susceptible to ATP inhibition under these conditions. These kinetic results strongly suggest that subunit self-association plays a significant role in the regulation of PFK.

## Discussion

The association–dissociation of rabbit muscle PFK has been the subject of intensive investigations (Parmeggiani et al., 1966; Ling et al., 1965; Leonard & Walker, 1972; Aaronson & Frieden, 1972; Hesterberg & Lee, 1981, 1982; Luther et al., 1983). These studies all agree that PFK does undergo association–dissociation and exists in various polymeric forms, but they do not all agree on the role of association–dissociation in the regulation of the enzyme. The focus of the discrepancy is whether these various polymeric forms are in rapid equilibrium as studied by sedimentation velocity.

One of the major conclusions of this study is that the various polymeric forms of *active* PFK are in rapid equilibrium regardless of the methods of purification. The use of heat and/or alcohol precipitation steps in the purification procedure alters the basic properties of PFK, leading to continuous generation of inactive enzyme. The inactive species can be fractionated from active PFK. The active PFK exhibits similar, but not identical, properties depending on the method of purification.

In examining the basic physical, chemical, and kinetic properties of active PFK, the most outstanding feature of the physical properties for active PFK is its ability to undergo a rapid association–dissociation. This conclusion is derived from the sedimentation behavior of active PFK, which demonstrates (a) an $\bar{s}_{20,w}$ independent of speed and (b) a time independence of $\bar{s}_{20,w}$ for a diluted sample of PFK. In every case tested, active

PFK exhibits these sedimentation properties. However, can these tests detect the presence of noninteracting or slow-interacting species of PFK? The results on unfractionated PFK as summarized in Table II clearly demonstrate that the tests employed are capable of differentiating PFK samples that are underoging rapid equilibrium from those that are not. Once establishing that active PFK oligomers are in a dynamic equilibrium, the self-association of active H-PFK, K-PFK, and L-PFK is characterized quantitatively. It is evident from the results summarized in both Figure 5 and Table IV that active PFK undergoes the same mode of association with a stoichiometry of $M_1 \rightleftharpoons M_4 \rightleftharpoons M_{16}$. While the association constants are similar, L-PFK demonstrates a weaker association.

The kinetic properties of active PFK were also examined. It is evident that active L-PFK shows a lower affinity for ITP as a substrate (Table III) as indicated by its $K_m$ of 151 $\mu$M, whereas active H-PFK and K-PFK exhibit a $K_m$ of $\sim$80 $\mu$M. However, this is the only observable difference in the kinetic behavior of active PFK prepared by the different purification procedures. The values for $K_{LATP}$ and $n$ are essentially identical for active PFK, whereas in unfractionated PFK samples these are significantly different depending upon the procedure of purification. Hence, one may conclude that the physical and kinetic behavior of active PFK prepared by these different methods are very similar. They behave as thermodynamic homogeneous samples that undergo association–dissociation in the same mode and have similar affinities for ATP and F6P. The loss of a peptide from L-PFK evidently affects the self-association and affinity for ITP quantitatively by about 2-fold.

Having established that active PFK does undergo a rapid association–dissociation, the issue of interest is the role of this subunit association in the regulation of PFK activity. In a series of studies (Hesterberg & Lee, 1980, 1981, 1982; Luther et al., 1983) we have shown quantitatively that PFK self-association is influenced by metabolites in a rapid manner and that it is linked to the conformational state of the enzyme. Thus, from a thermodynamic viewpoint the self-association of active PFK must play a role in its regulation. Upon examination of this proposal, it is logical to predict that any measurable parameter that is linked to the association–dissociation of PFK should change as a function of protein concentration. Hence, if the aggregation of PFK subunits is to play a role in the regulation of enzyme activity, the steady-state kinetic parameters of PFK such as $K_{m,app}$ should vary with protein concentration. This prediction is substantiated by the limited, but revealing, kinetic results shown in Table V. Although the quantitative linkage between kinetic parameters and subunit self-assembly has not yet been established, a qualitative rationale can be provided to describe the kinetic observations with respect to the role of subunit self-association.

Let us examine the rationale for the increase in $K_m$ value associated with a higher PFK concentration. The value of $K_m$ mainly reflects the affinity an enzyme has for its substrate. In a theoretical presentation, Cann & Hinman (1976) have elegantly shown that in a ligand-mediated self-association system the apparent affinity of a protein for a ligand can be higher than it actually is. In the PFK system, there is ample evidence to show that F6P enhances the self-association of PFK subunits (Hesterberg & Lee, 1982; Hill & Hammes, 1975; Lad et al., 1973). Hence, it is expected that at a lower protein concentration at which monomeric PFK predominantly exists, the presence of F6P should shift this equilibrium in favor of tetrameric PFK; thus, it leads to a lower value of $K_m$. The end result is an artifactually higher affinity for F6P. On the other hand, at a higher protein concentration a greater amount of tetrameric PFK will be present. The addition of F6P would not perturb this equilibrium to the same extent as that of the lower protein concentration. Hence, the measured affinity of PFK for F6P will approach that of the actual equilibrium constant which always assumes a lower affinity than the measured one in this scheme. Thus, the higher $K_m$ value observed at the higher protein concentration is consistent with the scheme of a ligand-facilitated self-association. Such a prediction has been convincingly substantiated by Na & Timasheff (1980) with the vinblastine–tubulin system.

In addition to an observable change in $K_m$ values, the Hill coefficient was shown to decrease also at a higher protein concentration. The Hill coefficient usually reflects the degree of cooperativity which depends on the affectiveness of a ligand in shifting an equilibrium. If the equilibrium is shifted to a greater extent, then a higher Hill coefficient will be expected. At lower PFK concentrations monomeric PFK will be the predominant species, and therefore, the monomer–tetramer equilibrium will be more significantly perturbed by F6P. Thus, this should lead to a higher value for the Hill coefficient under these conditions, as observed.

It has been reported that at high concentrations of ATP the dissociation of PFK is favored (Parmaggiani et al, 1966; Liddle et al., 1977). Hence, one could expect that in the presence of a greater amount of tetrameric PFK more ATP would be required to shift this equilibrium toward the inactive monomeric PFK. Therefore, the expected result is consistent with the observed higher value of $K_I$ for ATP at the higher PFK concentration. The same rationale can also be applied to the kinetic data of fractionated and unfractionated K-PFK and L-PFK as summarized in Tables I and III. Unfractionated PFK samples for these preparations contain inactive PFK; therefore, the effective concentration of active PFK is lower when compared to those of their corresponding fractionated samples. At a lower effective PFK concentration, it is expected that these samples should exhibit lower values for $K_I$ and higher Hill coefficients just as observed experimentally.

In conclusion, this study has established that the self-association of PFK does play a significant role in the regulation of the enzyme. The changes in kinetic parameters with protein concentration is consistent with theoretical considerations of an associating system (Nichol et al., 1967; Frieden, 1967). The challenge comes in establishing a quantitative linkage among kinetic, ligand binding, and structural data in a similar fashion as has been done with the rabbit muscle pyruvate kinase system (Oberfelder et al., 1984a,b).

**Registry No.** PFK, 9001-80-3; ATP, 56-65-5; ITP, 132-06-9; F6P, 643-13-0.

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