

Self-Association of Rabbit Muscle Phosphofructokinase at pH 7.0: Stoichiometry[†]

Lyndal K. Hesterberg and James C. Lee*

ABSTRACT: The self-association of rabbit muscle phosphofructokinase at pH 7.0 was investigated by velocity sedimentation. The process was demonstrated to be in a rapid, dynamic equilibrium. The concentration dependence of the weight-average sedimentation coefficient was monitored within the range of 10–750 $\mu\text{g/mL}$. The sedimentation properties of phosphofructokinase were analyzed by theoretical simulations for an associating system in rapid equilibrium. In the

absence of any ligands and at a temperature of 23 °C, the *simplest* computed model which gives the best fit between theoretical and experimental points can be described as progressive association of monomer \rightleftharpoons tetramer \rightleftharpoons 16-mer with apparent equilibrium constants $K_4 = 5.06 \times 10^5 \text{ (mL/mg)}^3$ and $K_{16} = 3.25 \times 10^{23} \text{ (mL/mg)}^{15}$. However, at 5 °C, the equilibrium was altered and can best be described as monomer \rightleftharpoons dimer \rightleftharpoons tetramer \rightleftharpoons 16-mer.

The self-association of rabbit muscle phosphofructokinase (PFK)¹ (ATP:D-fructose-6-phosphate 1-phosphotransferase) has been studied by many investigators by using such techniques as electron microscopy (Parmeggiani et al., 1966; Telford et al., 1975), X-ray diffraction (Paradies, 1979), inelastic light scattering (Goldhammer & Paradies, 1979), sedimentation equilibrium (Leonard & Walker, 1972), gel chromatography (Pavelich & Hammes, 1973; Aaronson & Frieden, 1972), and sedimentation velocity (Parmeggiani et al., 1966; Aaronson & Frieden, 1972; Ling et al., 1965; Leonard & Walker, 1972). The consensus is that PFK undergoes self-association in a complicated fashion. Neither the mode of association nor the thermodynamic parameters governing the association-dissociation equilibria have been systematically determined. There is, therefore, at present no unified model to describe the self-association reaction of rabbit muscle PFK.

Leonard & Walker (1972) reported that at pH 8.0, 20 °C, and protein concentration greater than 1 mg/mL the sedimentation patterns showed three peaks with $s_{20,w}$ values of 12, 18, and 30 S. These peaks were not completely resolved under their experimental conditions. With increasing protein concentration, the amount of protein in the fastest peak increased, whereas the amount of protein in the two slower peaks remained constant. They concluded that the sedimentation behavior was typical of a system of several components in a rapid reversible equilibrium of undetermined stoichiometry. At the same time, Aaronson & Frieden (1972) also reported the observation of three peaks at pH 8.0 and 10 °C. With increasing protein concentration, however, these authors reported that the areas encompassed by these peaks were linear functions of the total protein concentration, implying that these peaks represent the presence of noninteracting components. An attempt was made to separate the various polymeric forms of PFK by column chromatography. Fractions from the leading and trailing edges of the protein peak eluted from the column were subjected to sedimentation velocity analysis. The results revealed that these fractions exhibit different sedimentation profiles. Aaronson & Frieden (1972) concluded

that PFK appears to be a mixture of three components not in a rapid reversible equilibrium. These components represent three different monomeric forms of the enzyme with different propensity to self-associate.

Pavelich & Hammes (1973), utilizing frontal gel chromatography at 5 °C, studied the self-association of PFK over a pH range of 6.0–8.0 and at protein concentrations below 1 mg/mL. These authors reported that PFK undergoes dissociation from tetramers to dimers and probably to monomers. The dissociation was enhanced by lowering either the pH or the protein concentration. Due to the length of the experiment and the instability of PFK, up to 20% loss of enzyme activity was reported, thus preventing an accurate assessment of the mode of association and the equilibrium constants of the reaction.

It is evident that in spite of the potential importance of the association-dissociation of PFK in the regulation of this enzyme the quantitative determination of the physical parameters which govern this reaction has not been successful. A potential barrier is the instability of the enzyme, hence disqualifying essentially all techniques which require lengthy periods for data collection. Sedimentation velocity is an ideal technique to study unstable biological systems, and with the recent advent of the theoretical analysis of data it seems that the self-association of PFK can be studied by this technique, if the system were in rapid equilibrium.

In this paper, the results of a detailed investigation of the self-association of rabbit muscle PFK are reported. Within the limit of resolution by the technique of velocity sedimentation, PFK can be considered as a system undergoing rapid reversible equilibrium under the reported experimental conditions. A preliminary report of this work has been presented earlier (Hesterberg, 1980).

Materials and Methods

Rabbit muscle PFK was obtained by using the preparative procedure described previously (Hesterberg & Lee, 1980). The activity of PFK was monitored with the coupled enzyme system at pH 7.0 as modified by Hesterberg and Lee.² The specific activity of the PFK employed in this study was always

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¹ Abbreviation used: PFK, phosphofructokinase.

² L. K. Hesterberg and J. C. Lee, unpublished experiments.

above 300 units/mg. Prior to use, the enzyme was passed over a Sephadex G-25 column (0.9 × 4 cm) equilibrated with 25 mM Tris-CO₃, 1 mM EDTA, 6.0 mM MgCl₂, and 3.4 mM (NH₄)₂SO₄ (TEMA) buffer at pH 7.0. The concentration of PFK was determined spectrophotometrically with a Cary 118 spectrophotometer and an absorptivity of 1.07 mL/(mg-cm) at 280 nm (Hesterberg & Lee, 1980).

Velocity sedimentation experiments were performed in a Beckman Model E analytical ultracentrifuge equipped with an ultraviolet scanner, electronic speed control, and RTIC temperature control. Coated aluminum double-sector centerpieces (Kel-F; 12 mm) and sapphire windows were used in either an AN-D or an AN-F rotor at 60 000 and 52 000 rpm, respectively. The UV scanner was used to trace the protein boundary during sedimentation at 280 or 237.5 nm.

Weight-average sedimentation coefficients, \bar{s} , were determined from the centroid, a close approximation of the second moment, of the scanner-traced boundaries in a manner analogous to that of Arisaka & Van Holde (1979). The effect of radial dilution was not considered in these experiments. The observed weight-average sedimentation coefficients were normalized to standard conditions by correcting for solvent density and viscosity with a partial specific volume of 0.730 for PFK (Hesterberg & Lee, 1980).

For an associating protein system of n species in a rapid, dynamic equilibrium, the rate of sedimentation of the protein boundary is defined by the velocity of the square root of the second moment of the boundary and corresponds to the weight-average sedimentation coefficient, \bar{s} (Schachman, 1959), since

$$\bar{s} = \sum_i s_i C_i / \sum_i C_i \quad (1)$$

where s_i and C_i are the sedimentation coefficients and concentration of the i th species, respectively. In expressing each sedimentation coefficient, s_i , as a function of the total protein concentration, C , then

$$\bar{s} = \sum_i s_i^\circ (1 - g_i C) K_i C_1^i / \sum_i K_i C_1^i \quad (2)$$

where s_i° is the sedimentation coefficient of the i th species at infinite dilution, g_i is the nonideality coefficient, $C = \sum_i K_i C_1^i$, K_i is the equilibrium constant between any i -mer and the monomer, and C_1 is the monomer concentration.

Theoretical values of \bar{s} as a function of C_1 , s_i° , g_i , and K_i can be fitted to the observed weight-average sedimentation coefficients as a function of C ; thus, the best fitted curve can yield information on the physical parameters which govern the self-association reaction.

Results

Velocity Sedimentation of PFK. The self-association of PFK was studied by sedimentation instead of other physical techniques mainly due to the instability of the enzyme, which requires greater speed in collecting data before enzyme denaturation sets in, and the recent advance in the theory of analyzing sedimentation velocity data for an associating system. The assumption, however, is that the system under investigation should be in rapid, reversible equilibrium. It is, therefore, necessary to demonstrate that rabbit muscle PFK under the present experimental conditions does undergo rapid, reversible association.

When native PFK in TEMA buffer at pH 7.0 and 23 °C is subjected to sedimentation at 60 000 rpm, the sedimentation profile, expressed as derivative scans, is a function of the initial loading concentration of PFK, as shown in Figure 1. At a

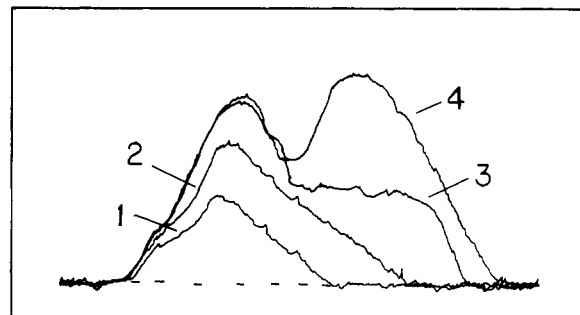


FIGURE 1: Sedimentation velocity profiles of PFK expressed as derivative scans in TEMA buffer at pH 7.00, 23 °C. Protein concentrations (in µg/mL) were the following: 1, 150; 2, 300; 3, 500; 4, 750. Profiles were traced with the derivative mode of UV scanner at 30, 32, 28, and 28 min for profiles 1–4, respectively, at 52 000 rpm.

concentration of 150 µg/mL, PFK sediments as a single, slightly skewed peak with $\bar{s}_{20,w} = 12.3$ S. At 300 µg/mL, the single peak sediments at about the same rate although the leading edge is now skewed. The formation of the second peak is apparent at 500 µg/mL, and the sedimentation coefficients of the slow and fast peaks are 12.2 and 18.7 S, respectively. When bimodality sets in, it appears that with increasing protein concentrations most of the protein is added to the faster peak as shown clearly by the sedimentation profile at 750 µg/mL. Such sedimentation behavior is characteristic of a self-associating system in rapid equilibrium relative to the length of the experiment (Gilbert, 1955). The presence of a bimodal sedimentation pattern implies that the stoichiometry of the reaction is ≥ 3 , as described by Gilbert (1955, 1959).

In an effort to establish that PFK does indeed undergo rapid reequilibration, two more additional tests were conducted. The $\bar{s}_{20,w}$ of PFK, over a range of protein concentrations, was determined at 52 000 and 60 000 rpm. If a self-associating system were in a slow equilibrium or if noninteracting or denatured components of the system were present, then $\bar{s}_{20,w}$ would be expected to change as a function of the angular velocity, ω , which in turn is directly related to the rotor speed. Results showed that the PFK solutions yield identical values of $\bar{s}_{20,w}$ at both speeds. It may, therefore, be concluded that within the limits of resolution and under the experimental conditions PFK undergoes rapid self-association.

Another study was conducted to probe at the equilibrium characteristics of PFK. The enzyme at an initial concentration of ≥ 1000 µg/mL was diluted at time zero to a concentration of 200–400 µg/mL. Aliquots of the diluted PFK solution were removed hourly and analyzed by velocity sedimentation at 60 000 rpm. If the association–dissociation reaction were governed by a slow equilibrium, a decrease in the $\bar{s}_{20,w}$ is expected with increasing time. The value of $\bar{s}_{20,w}$ should eventually reach a characteristic value at low protein concentrations. If the system were in a rapid equilibrium, the $\bar{s}_{20,w}$ at zero time should be characteristic of the lower protein concentration, and its value will not change over time. PFK solutions were subjected to such an analysis. The sedimentation properties of the enzyme were monitored from 0.5 to 6.5 h after the initial dilution. The results show that the $\bar{s}_{20,w}$ decreases to the value expected of PFK at the concentration studied by the time of the first analysis at 0.5 h. The value of $\bar{s}_{20,w}$ remains constant throughout the duration of the experiment, 6.5 h. This indicates that a slow equilibrium cannot exist under the experimental conditions. Apparently, there is no detectable denaturation or conversion into noninteracting components during the course of the test. Such an observation is consistent with the stability tests of the enzyme activity that

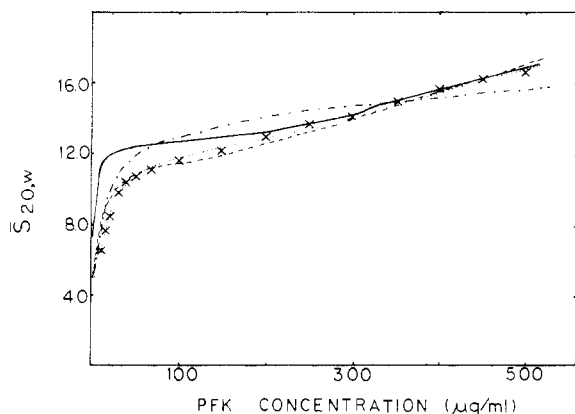
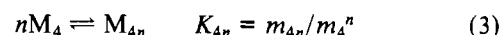


FIGURE 2: Theoretical fitting of the concentration dependence of the weight-average sedimentation coefficient, $\bar{s}_{20,w}$, of PFK in TEMA buffer at pH 7.00, 23 °C. Symbols and models for the theoretical curves are as follows: (···) $M = M_4 = M_{16}$ with $s_4^0 = 13.5$ S; (---) $M = M_4 = M_{16}$ with $s_4^0 = 12.4$ S; (-·-·-) $M = M_4 = M_8$ with $s_4^0 = 13.5$ S; (—) $M_2 = M_4 = M_{16}$ with $s_4^0 = 13.5$ S. (x) The average of multiple data sets.

PFK remains at least 95% active during this 6.5-h period² (Hesterberg & Lee, 1980). It appears, therefore, that a rapid, dynamic equilibrium involving all of the species of PFK is governing the self-association of the enzyme. The sedimentation velocity data can thus be analyzed quantitatively to determine the stoichiometry and thermodynamic parameters characteristic of the self-associating reaction.

The concentration dependence of the weight-average sedimentation coefficient of PFK in TEMA buffer at pH 7.0 and 23 °C was determined from 10 to 500 $\mu\text{g/mL}$, as shown in Figure 2. The range of protein concentration studied was chosen to mimic the concentration of the enzyme estimated in physiological conditions (Tornheim & Lowenstein, 1976). The lowest protein concentration studied is 10 $\mu\text{g/mL}$, below which the resolving capabilities of the UV scanner, as it is, do not yield accurate boundary tracings. It is evident that the value of $\bar{s}_{20,w}$ is a function of protein concentration, with a smaller value of $\bar{s}_{20,w}$ favored by lower PFK concentration. The extrapolated value of $\bar{s}_{20,w}$ at infinite dilution is about 5 S, indicating that the smallest species under the experimental condition is probably the monomeric unit of PFK at 80 000 molecular weight. The sharp increase in the observed value of $\bar{s}_{20,w}$ with increasing protein concentration implies that the aggregation of these monomeric units to higher orders of polymers may include species larger than the tetrameric unit, since the value of $\bar{s}_{20,w}$ at 500 $\mu\text{g/mL}$ is 16.7 S with apparently no indication of reaching an asymptotic value at such a protein concentration.

On the basis of the results from sedimentation studies, it may be concluded that PFK under the present experimental conditions undergoes rapid reversible self-association. The smallest species participating in the reaction is most likely the monomeric unit of PFK. The concentration dependence of $\bar{s}_{20,w}$ alone cannot distinguish between a model of indefinite self-association (Holloway & Cox, 1974) and a strong association of monomers to form higher orders of polymers (Cox, 1969). The presence of a bimodal sedimentation pattern, however, clearly indicates a monomer-polymer equilibrium. The sedimentation coefficient of the slow peak being 12.3 (Figure 1) implies that probably PFK undergoes a monomer \rightleftharpoons tetramer self-association. The presence of the second faster peak at higher protein concentrations (Figure 1) indicates that polymers of even higher orders are formed. Qualitatively, thus, self-association of PFK probably can be described as eq 3



where M_i denotes the i th aggregate, m_i is the molal concentration of each i th species, K_i is the equilibrium constant, and $n \geq 3$. Within the resolving capability of velocity sedimentation, all PFK species present are thermodynamically equivalent and capable of participating in the self-association reaction.

Model of Self-Association. In order to determine the physical constants characteristic of the self-association of PFK, a quantitative analysis was initiated by theoretical fitting of the concentration dependence of the weight-average sedimentation coefficient. Such an analysis has been successfully applied to the Mg^{2+} -induced and vinblastine-induced self-association of calf brain tubulin (Frigon & Timasheff, 1975a,b; Na & Timasheff, 1980a,b).

An attempt was made to fit the function of \bar{s} to the observed $\bar{s}_{20,w}$ obtained as a function of C by means of a nonlinear least-squares method (Magar, 1973). The procedure requires good initial estimates of the parameters s_i^0 , g_i , and K_i . Refined values of the parameters are then obtained by a reiteration scheme which employs the standard root mean square deviation, σ , as a criterion of convergence, where

$$\sigma = \frac{1}{n} \left[\sum_{j=1}^n (\bar{s} - \bar{s}_{\text{obsd}})_j^2 \right]^{1/2} \quad (4)$$

This approach works best when the minimum number of parameters, as defined in the function, are to be fitted. In order to apply such an approach to fit the data on the concentration dependence of $\bar{s}_{20,w}$ for PFK, reasonable estimates of s_i^0 and g_i are required. Under the present experimental conditions, the variation in any s_i across the sedimenting boundary is certainly less than 1%; therefore, $g_i = 0$ (Schachman, 1959; Chervenka, 1973).

The intrinsic sedimentation coefficient of the i th protein species, s_i^0 , for the various species considered can be obtained by using eq 5 where s_n^0 is the intrinsic sedimentation coef-

$$s_i^0 = s_n^0 \left(\frac{i}{n} \right)^{2/3} \quad (5)$$

ficient of the n th species. This relationship assumes an identical frictional ratio for all species (Cann, 1970; Nichol et al., 1964). Since the value of $\bar{s}_{20,w}$ at infinite dilution for PFK is 5.0 S, it might be assumed that it represents the smallest kinetic unit involved in the self-association reaction, i.e., $s_1^0 = 5.0$ S. With eq 5, the intrinsic sedimentation coefficients of dimers and tetramers can be calculated, yielding $s_2^0 = 7.9$ S and $s_4^0 = 12.6$ S. The estimated s_4^0 is in reasonable agreement with the literature value, which ranges from 12 to 15 S (Aaronson & Frieden, 1972; Leonard & Walker, 1972; Parmeggiani et al., 1966; Ling et al., 1965). Furthermore, it agrees well with the value of 12.4 S observed in active enzyme sedimentation (Hesterberg & Lee, 1980). As it is difficult to determine s_4^0 under the present experimental conditions, the theoretical modeling was attempted by using a range of s_4^0 between 12.5 and 14.0 S. Obviously, neither the assumption of spherical symmetry of all protein species nor the fact that $g_i = 0$ within the protein concentration range of this study is rigorously correct. Any attempts to incorporate the frictional properties of the various species by using either the s_i^0 or the g_i would unnecessarily complicate the model and the analysis. It must, however, be emphasized that in this analysis the actual magnitudes of the equilibrium constants

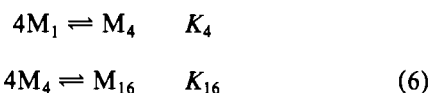
Table I: Summary of Fitting for Weight-Average Sedimentation Velocity Data, 23 °C

model	K_2^{app}	K_4^{app}	K_n^{app}	s_4°	σ
1-4-16	0	5.06×10^5	3.25×10^{23}	13.5	0.31
	0	2.98×10^6	5.23×10^{26}	12.4	2.18
1-4-8	0	2.00×10^6	7.04×10^{12}	13.5	5.80
2-4-16	1.0^b	468.7	2.17×10^{11}	13.5	19.2
	1.0^b	733.6	1.90×10^{12}	12.4	13.8
1-2-4-16	8.00×10^{-10}	5.03×10^5	3.34×10^{23}	13.5	0.31
	18.62	2.80×10^6	4.87×10^{26}	12.4	1.78

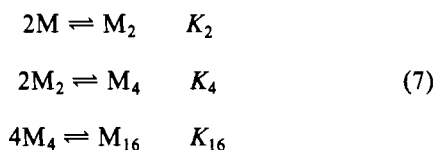
^a The values of s_i° remained constant for all species except s_4° . These values are the following: $s_1^\circ = 4.95$ S, $s_2^\circ = 7.6$ S, $s_8^\circ = 19.7$ S, and $s_{16}^\circ = 34.0$ S. ^b K_2^{app} assumes a value of 1.0 by definition.

are dependent on the assumed values selected for the parameters s_i° and g_i (Gilbert, 1963; Holloway & Cox, 1974).

With knowledge of the basic assumptions and by use of the estimated parameters as a first approximate, the fitting procedure was employed to estimate the mode of association and the equilibrium constants involved in the self-association of PFK at pH 7.0 and 23 °C. Typical results of the calculations for relevant combinations of the stoichiometry of self-association of PFK are shown in Table I. The calculated \bar{s} vs. C curves are compared in Figure 2 with the experimental points, where it is seen that the best agreement between the observed and calculated values is given by the model in which the self-association of PFK proceeds with a stoichiometry



The reaction scheme of eq 7 will fit equally as well as the sequential tetramerization model, as shown in eq 6 only when



the K_2^{app} is $\leq 8 \times 10^{-10}$ mL/mg, or insignificantly small. Precise values for K_2^{app} and K_3^{app} cannot be unequivocally determined with this technique alone. The values are based on data obtained at very low protein concentrations and are, therefore, vulnerable to the systematic errors inherent in these experimental conditions. Furthermore, the presence of significant experimental uncertainties does not enable one to completely exclude the presence of any dimers or trimers. However, on the basis of the results of data fitting, it may be concluded that the *simplest* model of the self-association of PFK at pH 7.0 and 23 °C is a sequential tetramerization with $K_4^{app} = 5.06 \times 10^5$ (mL/mg)³ and $K_{16}^{app} = 3.25 \times 10^{23}$ (mL/mg)¹⁵, and K_1 is by definition equal to 1.00. The values of S_i° which best fit the experimental data are 4.95, 7.6, 13.5, and 34.0 S for s_1° , s_2° , s_4° , and s_{16}° , respectively. Any deviations from these values either individually or in combination resulted in poor fitting of the data. It is interesting to note, as shown in Table I, that the experimental data do not fit well with s_4° of 12.4 S for the same model. The values of σ are much higher in both models tested.

The calculated s_4° is significantly higher than the $s_{20,w}^\circ$ of 12.4 ± 0.5 S reported for the active form of PFK (Hesterberg & Lee, 1980; Hesterberg, 1980). A potential source of this discrepancy is that sedimentation and diffusion of products during centrifugation were not included in calculating the results from active enzyme centrifugation. Cohen et al. (1967)

have estimated that this could introduce an error of 3–5% in the estimation of sedimentation coefficients. Another possibility is the difference in the solution constituents present in these different sedimentation experiments. In the active enzyme centrifugation, organic solvents were present to provide a stabilizing gradient. Such organic solvents may alter the hydrodynamic properties of the enzyme. Although it is possible that such an interaction might have taken place, it is unlikely because more than one solvent has been employed and consistent results were obtained. A third possibility is that in the presence of ligands such as substrates PFK undergoes a structural change during catalysis, leading to a molecule which is hydrodynamically more swollen. Such changes have been demonstrated most elegantly by Schachman and co-workers in the *Escherichia coli* aspartate transcarbamylase system (Gerhart & Schachman, 1968; Kirschner & Schachman, 1971, 1973; Howlett & Schachman, 1977). Furthermore, it was observed that while in the absence of any substrates a value of 13.5 S for s_4° gave the best fit, yet in the presence of either substrate a value of 12.4 S was required.² It seems, therefore, the apparent discrepancy in the sedimentation coefficient observed may indeed represent a structural change in PFK induced by the binding of either substrate. The molecular nature of such a proposed structural change is now being investigated.

Figure 3 depicts the mass distribution of PFK present in the different polymeric species as a function of the total protein concentration. The distribution was calculated in terms of the self-association of the mechanism described by eq 6 at 23 °C, pH 7.00. As depicted in Figure 3, the weight percentage of monomer decreases with increasing protein concentration. In the case of PFK tetramers, the weight percentage increases rapidly to a maximum of 86% at 190 mg/mL total protein and decreases very slowly at higher concentrations. On the other hand, the weight percentage of the 16-mer of PFK remains negligible below 150 mg/mL, but increases slowly with increasing protein concentrations above 150 mg/mL. Furthermore, at protein concentrations of 1 μ g/mL or lower, the concentration at which most routine steady-state kinetics are measured, PFK essentially is present in total as monomers. Since the active form is the tetramer, the binding of substrates must enhance the self-association dramatically to achieve such a state, a conclusion in agreement with that proposed previously (Hesterberg & Lee, 1980).

Pavelich & Hammes (1973) reported that at 5 °C and concentrations less than 0.2 mg/mL PFK is involved in a pH-dependent equilibrium between a dimer and a tetramer as studied by frontal gel chromatography. Such a conclusion was further substantiated by the results of a study at 4 °C employing cross-linkage of PFK with a bifunctional reagent (Lad & Hammes, 1974). It was concluded that the dimer is a fundamental unit for polymerization of the enzyme. These observations are in contrast to the present study. An insignificant amount of dimer is present under the experimental conditions reported in this study. Thus, it leads to the following question: What are the factors which lead to such apparently different observations? An obvious difference between this study and the studies reported by Hammes and co-workers is the temperature at which these experiments were conducted. The present study is conducted at 23 °C while Hammes and co-workers performed their experiments at 4–5 °C. It is conceivable that PFK may undergo a different mode of association at the lower temperature. The effect of temperature on the self-association of PFK was, therefore, examined.

Self-Association of PFK at 5 °C. Having determined the stoichiometry of the self-association of PFK at pH 7.0 and 23

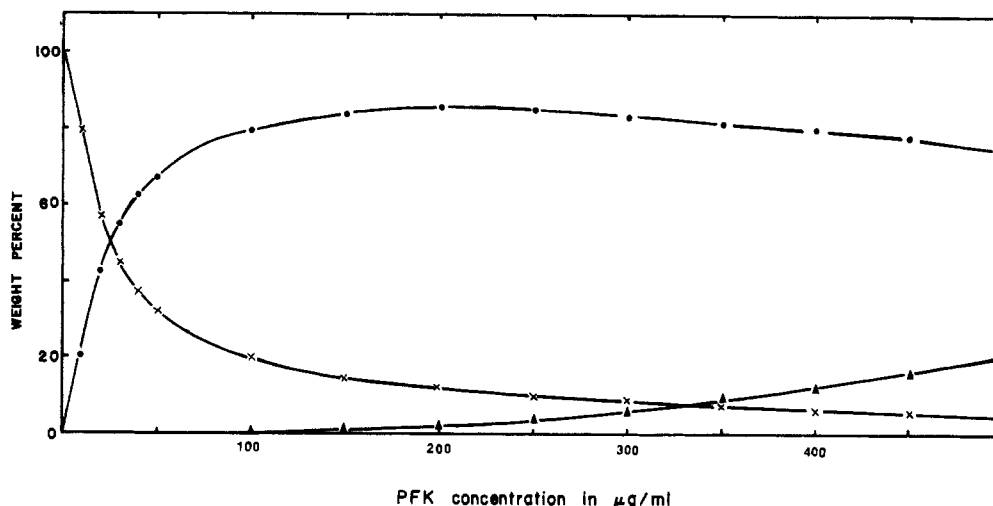


FIGURE 3: Mass distributions of PFK among polymeric species for a self-association system of $M = M_4 = M_{16}$ as a function of total protein concentration. Symbols and apparent equilibrium constants are the following: (X) monomer; (●) tetramer, $K_4^{app} = 5.06 \times 10^5$; (▲) 16-mer, $K_{16}^{app} = 3.25 \times 10^{23}$.

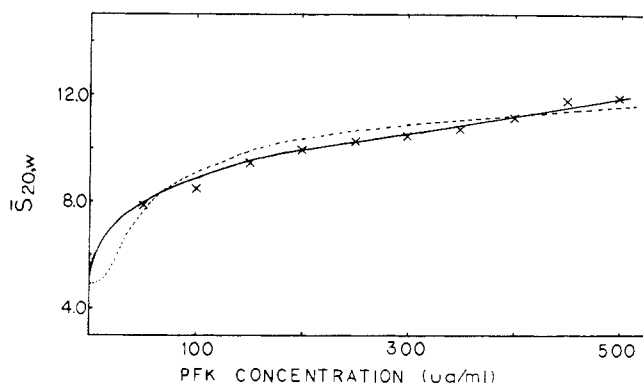


FIGURE 4: Theoretical fitting of the concentration dependence of the weight-average sedimentation coefficient, $\bar{s}_{20,w}$, of PFK in TEMA buffer at pH 7.0, 5 °C. Symbols and models for theoretical curves are as follows: (---) $M = M_4 = M_{16}$ with $s_4^\circ = 13.5$ S; (—) $M = M_2 = M_4 = M_{16}$ with $s_4^\circ = 13.5$ S. (X) Average of multiple data sets.

°C, it becomes desirable to gain insight into the basic parameters which govern the reaction. The concentration dependence of $\bar{s}_{20,w}$ between 25 and 500 $\mu\text{g/mL}$ PFK in TEMA buffer at pH 7.0 ± 0.1 was studied at 5 ± 0.1 °C. The uncertainty in the pH is mainly due to the instability of Tris- CO_3 buffer as a function of temperature.

The concentration dependence of $\bar{s}_{20,w}$ at 5 °C is shown in Figure 4. The equilibrium constants of the polymerization reaction were calculated by theoretical simulation as described previously. At 23 °C, the best fit of the data could be obtained with the model of monomer \rightleftharpoons tetramer \rightleftharpoons 16-mer. At 5 °C, however, it became difficult to fit the data with such a model. By consideration of the presence of dimers, i.e., monomer \rightleftharpoons dimer \rightleftharpoons tetramer \rightleftharpoons 16-mer, the theoretical fit of the experimental data improves dramatically as shown in Figure 4. Furthermore, the model of dimer \rightleftharpoons tetramer \rightleftharpoons 16-mer does not yield a good fit as shown in Table II, which also shows the calculated values for the apparent equilibrium constants K_2^{app} and K_4^{app} . The values of K_2^{app} decreased with increasing temperature and must approach zero at 23 °C. This can account for the satisfactory fitting of the data with the sequential tetramerization model at high temperatures. The values of K_4^{app} , as shown in Table II, remain essentially constant throughout most of the temperatures studied. The small changes observed in the K_4^{app} are not sufficient to account for

Table II: Summary of Fitting for Weight-Average Sedimentation Velocity Data, 5 °C

model	K_2^{app}	K_4^{app}	K_n^{app}	s_4°	σ
1-2-4-16	239.9	5.69×10^5	3.12×10^{23}	13.5	0.003
	135.0	5.50×10^5	1.03×10^{22}	12.4	0.98
1-4-16	0	7.74×10^3	4.23×10^{14}	13.5	2.21
	0	1.50×10^4	4.77×10^{15}	12.4	0.53
2-4-16	1.0 ^b	4.30	3.10×10^3	13.5	0.54

^a The values of s_i° remained constant for all species except s_4° . These values are the following: $s_1^\circ = 4.95$ S, $s_2^\circ = 7.6$ S, $s_8^\circ = 19.7$ S, and $s_{16}^\circ = 34.0$ S. ^b K_2^{app} assumes a value of 1.0 by definition.

the observed changes in the concentration dependence of $\bar{s}_{20,w}$ shown in Figure 4. Thus, it appears that the primary effect of temperature below 23 °C is on K_2^{app} . The weight percent distribution of various species at 5 °C is shown in Figure 5. It is evident that at protein concentrations below 20 $\mu\text{g/mL}$ the predominant species are monomers and dimers, with dimers and tetramers becoming the major species at higher concentrations. It is, therefore, to be expected at such conditions that the predominant species observed by Hammes and co-workers are the dimers and tetramers (Pavelich & Hammes, 1973; Lad & Hammes, 1974).

Apparently, the major effect of lowering the temperature is to enhance the formation of dimers, whereas the assembly to higher orders of aggregates is not significantly altered. Tentatively, these observations imply that the dimerization of PFK is enthalpy driven and the self-association of tetramers and 16-mers is entropy driven.

Discussion

Rabbit muscle PFK at pH 7.0 has been demonstrated in this study to be a system in rapid, dynamic equilibrium. The mode of association is sensitive to temperature, with low temperature favoring the formation of dimers in addition to tetramers and 16-mers, which are the predominant species at higher temperatures. At present, no information can be provided for the stoichiometry of the highest order of aggregation. In fact, 16-mer is most likely not the end product under the experimental conditions.

As this study is one of the first to employ theoretical analysis of sedimentation velocity data which have been accumulated

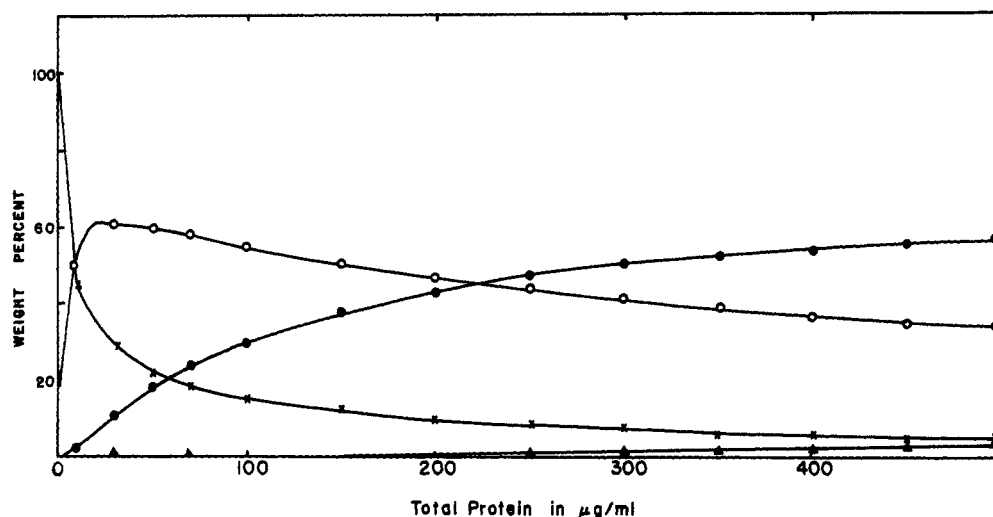


FIGURE 5: Mass distribution of PFK among polymeric species for a self-association system of $M \rightleftharpoons M_2 \rightleftharpoons M_4 \rightleftharpoons M_{16}$ as a function of concentration. Symbols and apparent equilibrium constants are the following: (x) monomer; (o) dimer, $K_2^{app} = 239.9$; (●) tetramer, $K_4^{app} = 5.69 \times 10^5$; (▲) 16-mer, $K_{16}^{app} = 3.12 \times 10^{23}$.

by the use of a UV scanner, a discussion of some of the difficulties encountered in the data collection and the assumptions made during analysis is in order.

One of the initial difficulties encountered in this study was the behavior of the sedimentation boundary at protein concentrations below $20 \mu\text{g/mL}$. The signal obtained with the UV scanner within this protein range is very small, but, of more significance, the signal to noise ratio was much lower than that at higher protein concentrations. The initial scanner tracings of the sedimenting protein boundary reveal sharp, well-defined tracings, but as time progressed the rate of diffusion was sufficient so that the traced boundary was not discernible from the noise in the base lines of the tracing. This instability of the boundary limited that data collection to the initial 24–28 min. This minimizes any effects of radial dilution, but the inherent error in the collection of data at protein concentrations below $20 \mu\text{g/mL}$ is significantly larger than those at higher protein concentrations.

An additional difficulty encountered was the nonlinearity of the scanner-derived boundary and derivative tracings at different range settings. This appears to be inherent in the UV scanner. The problem arises when one attempts to compare scanner tracings or derivative curves obtained at one protein concentration and scanner range setting to tracings and curves obtained at different protein concentrations and scanner range settings. The scanner range settings must vary as protein concentrations vary both to accommodate the increased signal and to provide the optimum resolution of the boundary. Comparison between ranges is possible only after point by point corrections are made to ensure linearity of the scanner tracings with protein concentration.

The ideal situation would be to have a sharp boundary existing between the two linear base lines to ensure accurate analysis of the scanner-traced boundaries. This, however, does not occur with all protein concentrations throughout the experiment. In general, scanner tracings obtained early in the centrifuge experiment reveal very sharp protein boundaries, but these boundaries have not sedimented sufficiently to make the choice of the base line unequivocal. Scanner tracings obtained late in the centrifuge run reveal a diffuse protein boundary complicated with a very short column at the plateau region, making the determination of the plateau region very difficult. In general, the optimum conditions existed only in the intermediate time scans during a centrifuge experiment

while the protein boundary remained reasonably sharp with sufficient resolution of both base lines. The length of time during which these optimum conditions exist varies with the protein concentration and the choice of rotor speed. The choice of base lines is inherently difficult when utilizing the UV scanner, but when the protein being studied is capable of self-association additional complications become apparent. As in the case of PFK, any protein system that undergoes a rapid association–dissociation reaction with a stoichiometry of ≥ 3 must at some protein concentration, depending on the equilibrium constant, exhibit a bimodal sedimentation profile (Gilbert, 1955). At protein concentrations slightly greater than this critical concentration, there should be a skewing of the protein peak, indicating the formation of a faster moving peak, as shown in Figure 1. The boundary of the sedimenting protein traced by the scanner reveals, however, only a boundary with a shoulder on the leading edge. A second sharp boundary will not become apparent except at higher loading concentrations. There exists, therefore, a range of protein concentrations within which the determination of base lines at the plateau region becomes very difficult, thus introducing a significant amount of error by yielding artificially low values for $\bar{s}_{20,w}$. The range in this study occurs between 250 and $350 \mu\text{g/mL}$. Only if this complication is anticipated can appropriate base lines be chosen, and this source of error minimized.

In the analysis of the association–dissociation equilibrium of PFK, three major assumptions were made: (1) the nonideality coefficient, g_i , for all species is negligible within the concentration range studied; (2) all species of PFK exhibit an identical frictional ratio; (3) the effect of radial dilution is insignificant. Let us examine the effects on the results reported if any of these assumptions were not valid.

If the nonideality coefficient, g_i , was significant for the protein species, its effect would be the greatest at the higher protein concentrations and would be minimized at the lower concentrations. This would manifest itself in artificially low $\bar{s}_{20,w}$ values at the higher protein concentrations. Therefore, one might expect that the rate of increase in the value of $\bar{s}_{20,w}$ as a function of protein concentration would be greater if the nonideality were significant. The stoichiometry of the association would probably not change, but the equilibrium constants reported here would be lower than those calculated if g_i were taken into consideration. As outlined earlier, within the protein concentration range of this study, it is reasonable

to assume that thermodynamic nonideality has no significant effect on the experimental results.

The identical frictional ratio assumed for all species of PFK is another factor which, if incorrect, could influence the results reported in this paper. As the axial ratio of the protein increased, the intrinsic sedimentation coefficient of the protein will decrease. The effect of this on the computer modeling can be estimated but remains largely conjecture. The best fit of the data (Figure 2) is still given by species with $s^{\circ}_{20,w} = 34.0, 13.5$, and 4.95 S. It is unlikely that the 13.5 S and 4.95 S species can be due to any species other than the tetramer and monomer, respectively. However, the higher order aggregate could indeed have a stoichiometry greater than 16, so long as the $s^{\circ}_{20,w}$ of the species would be 34 S. This is only possible if a large asymmetry existed. As the structure of this higher order aggregate has not been fully characterized, the simplest approach to the analysis of the association is required. Once the characterization of the higher order polymeric species is known, the appropriate hydrodynamic frictional coefficients can be determined and the data reanalyzed in light of the new information. The conclusion presented in this paper represents the simplest set of conditions under which the analysis could be performed.

As stated earlier, the effects of radial dilution on the sedimentation boundary were not considered in this study. For a homogeneous system, neglecting radial dilution may lead to an overestimation of the sedimentation coefficient by as much as 10% (Lauffer, 1944; Trautman et al., 1954; Baldwin, 1957). Furthermore, the fractional increase in the sedimentation coefficient is directly proportional to the initial concentration (Cheng & Schachman, 1955). The effect can be detected by the upward curvature of the plot of $\ln \bar{R}$ vs. time, where \bar{R} is the radial distance of the boundary. On the other hand, for a system which undergoes association-dissociation, radial dilution would shift the equilibrium toward dissociation, leading to a downward curvature of the plot of $\ln \bar{R}$ vs. time. The plots included in this study show no consistent curvature and always yield correlation coefficients of ≥ 0.99 . Thus, the assumption that radial dilution has no significant effect on the results reported in this study is apparently valid, although it is conceivable that the observed linearity is the consequence of a compensatory effect of the two phenomena described.

In conclusion, the results of this investigation show that PFK undergoes reversible association-dissociation, a process demonstrated to be rapid and dynamic. The simplest model describing the process is monomer \rightleftharpoons tetramer \rightleftharpoons 16-mer at temperatures higher than 20°C , whereas at 5°C the formation of dimers is favored. The results of this analysis can be further tested by utilizing theoretical simulations of the sedimenting protein boundary as outlined by Cox (1971), and testing is presently in progress. As the basic physical parameters which govern the self-association of PFK are being defined, the effects of ligands on the equilibrium of this reaction can now be investigated quantitatively, hoping to elucidate the mechanism involved in the regulation of the in vitro polymerization-depolymerization of the enzyme.

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