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## Regulation and quaternary structural changes in rabbit muscle phosphofructokinase

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Subunit assembly plays a significant role in the regulation of rabbit muscle phosphofructokinase (PFK), although conformational changes and post-translational modifications have also been implicated to regulate the enzyme activity. In the absence of high-resolution structural information, the three-dimensional arrangements of subunits in the rabbit muscle PFK in its active and inactive states are not known. Hence, a systematic study is initiated, and phosphorylation of PFK subunit is employed as a probe for the structure-function correlation of the enzyme. The self-association of the phosphorylated and dephosphorylated PFK was monitored by sedimentation velocity at pH 7.0 and 23°C. Results show that both the phosphorylated and dephosphorylated forms of PFK exhibit the same mechanism of assembly. The secondary structures of both forms of PFK were monitored by circular dichroism (CD) as a function of protein concentration ranging from 20 to 2000 µg/ml. Results show that there is no detectable difference in the structure under all experimental conditions. The accessibility of tryptophan to solvent was monitored by fluorescence quenching within the same range of protein concentration. Results show that the fluorophores are more accessible to the quencher at higher protein concentrations. Hence, post-translational modification and subunit association do not induce significant structural change in PFK subunit, although the accessibility of tryptophan residues is altered with oligomer formation. Furthermore, sedimentation and CD studies show that the activation of PFK by substrate includes no detectable modification in secondary/tertiary structure but a quaternary structural change, and the local environments of some, if not all, of the tryptophan residues are less accessible to solvent. Hence, the change in sedimentation behavior between the active and inactive tetrameric PFK is due to a rearrangement of subunit-subunit interactions. In order to correlate the physical properties of PFK to the regulatory behavior of enzyme activity, the steady-state kinetics were investigated under the same experimental conditions. In conditions where enhancement of self-association is observed, the kinetic behavior reflects activation of the enzyme. Hence, this correlation between subunit assembly and the regulation of enzyme activity in PFK must reflect an intrinsic property of the muscle enzyme.

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Abbreviations: PFK, in vivo phosphorylated phosphofructokinase; de-PFK, dephosphorylated phosphofructokinase; F6P, fructose 6-phosphate; ATP, adenosine 5'-triphosphate; DTT, dithiothreitol; TEMA buffer, 25 mM Tris-CO<sub>2</sub> with 1 mM EDTA, 6 mM MgCl<sub>2</sub>, and 3.0 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> at pH 7.0.

### 1. Introduction

The molecular mechanism of allosteric regulation of rabbit muscle phosphofructokinase (PFK) is very complex and has been reported to involve subunit self-association [1], changes in the hydrodynamic properties [2,3], conformational changes [4,5], and post-translational modification [6,7]. Although the enzyme has been the subject of inten-

sive investigation for many years, the coordination of these different levels of structural changes with respect to the basic function of PFK has not been clearly established. One of the reasons is the lack of crystallographic information on the muscle enzyme, although the structures of PFK from both *Bacillus stearothermophilus* and *Escherichia coli* have been reported as a consequence of the elegant studies by Evans and co-workers [8–10]. However, the bacterial enzymes are smaller proteins which consist of four identical subunits with a molecular weight of 34 000 per subunit. This is less than half of the size of the muscle enzyme which has a subunit molecular weight of 83 000 [1]. Since the basic nature of the bacterial enzymes is so different from that of the mammalian one, there is no a priori reason to believe that these enzymes should share any mechanism of regulation.

Although in earlier studies from this laboratory, it has been shown that subunit self-association is related to the regulation of muscle PFK activity, it is not certain if this relationship holds under all experimental conditions. In this present case, phosphorylation of muscle PFK was employed as a probe to perturb both the structure and kinetic behavior of rabbit muscle PFK. Höfer and co-workers [6,7] have shown that muscle PFK can undergo phosphorylation/dephosphorylation as a function of muscle activity; hence, this post-translational modification may serve as an additional means of regulation. While no difference in maximum catalytic activity has been observed between the two forms, there are detectable differences in the allosteric kinetic properties [11–14]. Also, upon phosphorylation, PFK has a higher affinity for F-actin [13]. Thus, it is proposed that phosphorylation/dephosphorylation of muscle PFK acts as a means by which this key glycolytic enzyme is compartmentalized to cellular components where the flux of energy is required. This proposal is well supported by a variety of physiological observations [15–22]. Hence, this post-translational modification affects not only the allosteric regulatory behavior of the enzyme but also the interaction of PFK with other cellular components. The exact mechanism(s) in which phosphorylation exerts its effect on the kinetic properties of PFK is not known. These may include local

conformational changes, significant movements of protein domains, subunit self-association, or a combination of the above modes of action. Hence, a study was initiated to investigate the effect of phosphorylation. In this paper, subunit interaction and structural information of PFK and de-PFK are reported. Preliminary results of this study have been reported [23,24].

## 2. Materials and methods

ATP, F6P, DTT, the catalytic subunit of 3',5' cAMP-dependent protein kinase, and bovine intestine alkaline phosphatase (type VII) were purchased from Sigma. Aldolase, glycerol-3-phosphate dehydrogenase/triosephosphate isomerase and NADH were obtained from Boehringer-Mannheim. These were all used without further purification. PFK was purified, stored, and assayed as previously described [1].

In vivo phosphorylated PFK was prepared by the method of Höfer and Sorenson-Ziganke [7] as described by Hesterberg [25]. In vitro phosphorylation of PFK was accomplished by the procedure reported by Kitajima et al. [11] and adopted by Luther and Lee [13]. PFK was dephosphorylated by the method of Foe and Kemp [14], as reported by Luther and Lee [13]. The amount of phosphate covalently bound to PFK was determined by the procedure of Hasegawa et al. [26] using phosphoserine as the standard.

PFK protein concentrations were determined spectrophotometrically. The values of the wavelength and absorption coefficient were 280 nm and  $1.07 \text{ l g}^{-1} \text{ cm}^{-1}$  respectively [27].

In all experiments, PFK was equilibrated in the appropriate buffer by passage through a Sephadex G-25 column ( $1.2 \times 8.5 \text{ cm}$ ). PFK was assayed for activity and regulatory properties as previously described [13].

Sedimentation velocity studies were performed and analyzed according to previously published procedures [27]. The rate of sedimentation of the protein boundary is expressed as the weight-average sedimentation coefficient,  $\bar{S}$  [28], since  $\bar{S} = \sum S_i C_i / \sum C_i$  where  $S_i$  and  $C_i$  are the sedimen-

tation coefficients and concentration of the  $i$ -th species, respectively. Weight-average sedimentation coefficients were normalized to standard conditions by correcting for solvent density and viscosity. Experiments were performed in TEMA buffer at pH 7.0 and 23°C. For all experiments, Kel-F-coated aluminium double-sector center-pieces with sapphire windows were used. The apparent partial specific volume of PFK is 0.730 [2].

Acrylamide quenching experiments were conducted in an effort to provide additional information on the accessibility of the tryptophan residues in PFK as a function of protein concentration and ligation. Interaction between acrylamide in the solvent and tryptophan residues in the protein should be dependent upon the accessibility of these fluorophores. Since the protein is in a dynamic equilibrium, the degree to which a specific residue is quenched depends upon the time-average degree of exposure of that residue. If a conformational change occurs, this may lead to a change in the accessibility of the fluorophore, and thus the acrylamide concentration dependence of quenching may be affected. Acrylamide quenching experiments were performed in TEMA buffer and the data were analyzed by the Stern-Volmer equation [29];

$$\frac{F_0}{F} = 1 + K_Q[Q] \quad (1)$$

where  $F_0$  and  $F$  are the fluorescence emission intensities in the absence and presence of quencher, respectively;  $K_Q$  denotes the collisional quench constant, and  $[Q]$  the concentration of quencher. Since there are multiple fluorophores in PFK,  $K_Q$  is approximately the weight-average  $K_Q$  [30]:

$$K_Q = \sum_{i=1}^n K_i/f_i \quad (2)$$

where  $K_i$  is the dynamic constant for fluorescent component  $i$ ;  $f_i$  is the fractional contribution of component  $i$  to the fluorescence.

The conformation of PFK was also monitored by CD using a Cary model 61 spectropolarimeter. The spectra were routinely recorded from 350 to 200 nm. Overlapping spectra were obtained with

0.1- and 0.01-cm fused silica cells. A value of 114 was used for the mean residue weight of PFK in the calculation of ellipticities  $[\theta]$ . All runs were performed at approx. 23°C.

### 3. Results

The phosphorylated and dephosphorylated PFK contained the same amount of phosphate as reported previously [13], namely,  $0.7 \pm 0.07$  and  $0.01 \pm 0.01$  mol phosphate per mol PFK subunit for the *in vivo* phosphorylated and dephosphorylated forms of PFK, respectively.

Having prepared phosphorylated and dephosphorylated PFK, it is of interest to identify the physical state(s) of these forms in order to elucidate further the role of phosphorylation/dephosphorylation in the molecular mechanism(s) of modulating PFK activity. Native PFK has been conclusively demonstrated to undergo self-association; hence, the ability of the enzyme to aggregate was monitored by sedimentation velocity. Experiments were conducted at pH 7.0 and 23°C in TEMA buffer within the protein concentration range of 0.025–2.0 mg/ml. Under all experimental conditions and for all PFK forms employed, the systems were tested for rapid, dynamic equilibrium by reference to criteria previously adopted, namely, measurement of  $\bar{s}_{20,w}$  as a function of  $\omega^2$ , and independence of  $\bar{s}_{20,w}$  as a function of time of dilution from a stock solution of higher protein concentration. On the basis of these criteria, it was established that the self-association reaction is rapid, and that the data are amenable to analysis to obtain equilibrium constants and stoichiometry. When phosphorylated and dephosphorylated PFK forms were subjected to sedimentation analysis, it was observed that the values of  $\bar{s}_{20,w}$  for phosphorylated PFK below the concentration of 0.4 mg/ml were lower than that of the dephosphorylated enzyme, as shown in fig. 1. At concentrations above 0.4 mg/ml, the trends are reversed, indicating that either the phosphorylated PFK has a different mode of association or that the equilibrium constants are different. Upon *in vitro* rephosphorylation of the dephosphorylated form, PFK exhibits a sedimentation behavior identical

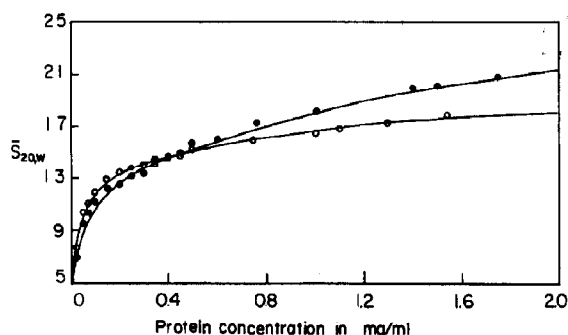


Fig. 1. Relation between weight-average sedimentation coefficient and PFK concentration in TEMA buffer at pH 7.0 and 23°C. Samples: (●) phosphorylated PFK, (○) dephosphorylated PFK. Lines represent the theoretical fit of the experimental data with the association model of  $M_1 \rightleftharpoons M_4 \rightleftharpoons M_8 \rightleftharpoons M_{16}$ . The data points represent the average of multiple data sets.

to that of the *in vivo* phosphorylated form, indicating that the differences in the observed  $\bar{s}_{20,w}$  are due to the process of phosphorylation and are reversible.

The results from sedimentation experiments were further analyzed by theoretical calculations to obtain stoichiometry and equilibrium constants of the reaction since

$$\bar{s} = \sum_i S_i^\circ (1 - g_i C) K_i C_1^i / \sum_i K_i C_1^i \quad (3)$$

where  $S_i^\circ$  denotes the sedimentation coefficient of the *i*-th species at infinite dilution,  $g_i$  the non-ideality coefficient,  $C = \sum_i K_i C_1^i$ ,  $K_i$  the equilibrium constant between any *i*-mer and the monomer and  $C_1$  the monomer concentrations. The procedure requires initial estimates of the parameters  $S_i^\circ$ ,  $g_i$  and  $K_i$ .

The values of  $S_i^\circ$  for the various species involved in the reaction can be obtained by using eq. 4

$$S_i^\circ = S_n^\circ (i/n)^{2/3} \quad (4)$$

where  $S_n^\circ$  is the sedimentation coefficient of the *n*-th species at infinite dilution. The various  $S_i^\circ$  values used in the fitting are  $S_1^\circ = 5.4$  S,  $S_2^\circ = 8.5$  S,  $S_4^\circ = 13.5$  S,  $S_8^\circ = 21.4$  S and  $S_{16}^\circ = 34.0$  S. The rationale for adopting these values is twofold, namely, a tetramer with  $S_4^\circ = 13.5$  S was detected directly in an enzymatically inactive condition [3]

and by using six standard globular proteins of known molecular weight and  $S^\circ$  values, it can be shown that monomeric PFK of molecular weight 83000 should sediment as a particle of  $S = 5.4 \pm 0.3$  S, a value in excellent agreement with the estimated value based on  $S_4^\circ = 13.5$  S. An implicit assumption in this calculation is that there is no structural change among all protein species and an identical frictional ratio for all species. This assumption is probably valid (see structural studies reported in this paper).

In earlier reports from this laboratory [3,13,27], the studies were limited to protein concentrations below 0.5 mg/ml. It was assumed that  $g_i = 0$ ; however, in this study, the concentration range of PFK has been extended to at least 2 mg/ml. The appropriate value for  $g$  was determined by fitting the data above 2 mg/ml of protein to the relation [28]

$$S = S^\circ (1 - gC) \quad (5)$$

resulting in a value of  $g = 0.02$  ml/mg.

In analyzing the data to determine the stoichiometry and equilibrium constant, it became evident that under certain conditions, more than one mode can describe the data equally well. Hence, the following set of rules was adopted to aid in assessing the reliability of data analysis.

(1) Use either the standard root mean square deviation ( $\sigma$ ) or sum of squares of residuals (SS) to select the mode of association. The lower the value of  $\sigma$  or SS, the better are the calculated data compared with those determined experimentally [27,31,32].

(2) If two or more modes of association have equal or very similar  $\sigma$  values, then the mode with the smallest number of species is chosen.

(3) If rules 1 and 2 cannot allow differentiation between modes of association, then only those equilibria with consistent values for apparent association constants  $K_i^{\text{app}}$  are included in the final data analysis.

The sedimentation data for the phosphorylated form of PFK were analyzed for different modes of association. The results are summarized in table 1. By analyzing the data that cover the whole concentration range of 0–2 mg/ml, it is obvious that the mode with a stoichiometry of 1:4:8:16 rep-

Table 1

Summary of fitting for weight-average sedimentation velocity data in TEMA buffer at pH 7.0 and 23°C

Stoichiometry	$K_2$ (ml/mg)	$K_4$ (ml/mg) <sup>3</sup>	$K_8$ (ml/mg) <sup>7</sup>	$K_{16}$ (ml/mg) <sup>15</sup>	SS
(A) Phosphorylated PFK					
1:4:16		$5.4 \pm 0.5 \times 10^5$		$5.9 \pm 2 \times 10^{22}$	32
1:4:16 <sup>a</sup>		$4.8 \pm 0.5 \times 10^5$		$9.4 \pm 3 \times 10^{22}$	0.06
1:4:8		$1.3 \times 10^5$	$3.3 \times 10^{10}$		117
1:2:4:8	428	$1.3 \times 10^6$	$4.1 \times 10^{13}$		80
1:4:8:16		$3.7 \pm 0.4 \times 10^5$	$6.3 \pm 2 \times 10^{10}$	$1.7 \pm 0.4 \times 10^{22}$	5
1:4:8:16 <sup>a</sup>		$4.6 \pm 0.5 \times 10^5$	$1.1 \pm 0.4 \times 10^{10}$	$7.8 \pm 1.5 \times 10^{22}$	0.01
1:2:4:8:16	37	$8.6 \times 10^5$	$4.6 \times 10^{11}$	$6.5 \times 10^{23}$	3
(B) Dephosphorylated PFK					
1:4:16		$4.6 \times 10^5$		$6.9 \times 10^{21}$	52
1:4:8		$2.0 \times 10^5$	$4.7 \times 10^{10}$		20
1:2:4:8	30 ± 5	$5.1 \pm 0.5 \times 10^5$	$2.3 \pm 0.4 \times 10^{11}$		4
1:4:8:16		$2.6 \pm 0.3 \times 10^5$	$3.7 \pm 0.3 \times 10^{10}$	$4.2 \pm 2 \times 10^{20}$	2
1:2:4:8:16	11 ± 3	$3.5 \pm 0.3 \times 10^5$	$9.2 \pm 0.7 \times 10^{10}$	$2.2 \pm 2 \times 10^{10}$	4

<sup>a</sup> Data within the protein concentration range 0–0.5 mg/ml were analyzed.

resents the data well, since the value for SS is 5, which is significantly better than that for other combinations of stoichiometries. This conclusion differs from that of an earlier report from this laboratory under the same experimental conditions [27]. Previously, it was concluded that the stoichiometry was 1:4:16. The only obvious difference between the two studies is the protein concentration range, i.e., 0–0.5 mg/ml in the earlier study and 0–2 mg/ml in the present investigation. To identify the cause of this discrepancy, the current data within the range 0–0.5 mg/ml were analyzed. The results show that both modes of 1:4:16 and 1:4:8:16 fit the data equally well, as shown in table 1. The values of  $K_4$  and  $K_{16}$  are essentially identical, regardless of the mode chosen in data fitting. Thus, application of rule 2 would favor the choice for the mode with the least components, i.e., 1:4:16, the same conclusion derived from the earlier study. It is apparent that when the study was limited to the low concentration range, there is not enough octamer present in the solution to help differentiate between the modes of 1:4:16 or 1:4:8:16. This is a direct consequence of the value of  $K_8$ . In order to ensure that the present conclusion reflects an intrinsic property of the PFK system under these experimental conditions, more data were collected up to

9.2 mg/ml. The same conclusion can be derived irrespective of whether the chosen range of data is from 0 to 2 or from 0 to 9.2 mg/ml; hence, it may be concluded that a finite stoichiometry can be defined if sufficient amounts of the major species are present within the concentration ranges studied. The accuracy in the determination of  $K_4$  is much better than that of  $K_{16}$ , e.g.,  $\pm 26\%$  vs  $\pm 140\%$  deviations from the mean. This is a consequence of the respective value of  $K_i$  and the concentration range studied. Obviously, with higher values of  $K_i$ , the  $i$ -th component will be present in more significant amounts over a wider concentration range, and the experimental results will in turn enable a more accurate evaluation of  $K_i$ . This conclusion is clearly demonstrated by the plot of weight percentage of oligomers as a function of protein concentration, as shown in fig. 2. It is quite evident that a significant amount of  $M_{16}$  is present only at PFK concentrations greater than 0.5  $\mu\text{g/ml}$  and  $M_8$  is never present in high percentage throughout the concentration range.

The sedimentation data for the dephosphorylated form of PFK were analyzed in a similar manner, and the results are summarized in table 1. By applying rule 1, it becomes evident that the mode of association that fits the data best is 1:4:8:16, the same as that of the phosphorylated

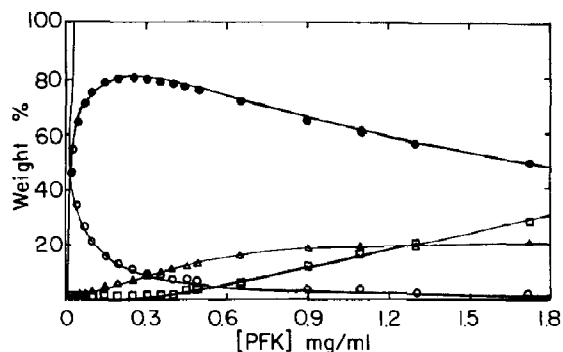


Fig. 2. Mass distribution of PFK among oligomeric species as a function of total protein concentration. Apparent equilibrium constants: (○) monomer; (●) tetramer,  $K_4^{app} = 4.6 \times 10^5$  (ml/mg)<sup>3</sup>; (△) octamer,  $K_8^{app} = 1.1 \times 10^{10}$  (ml/mg)<sup>7</sup>; (□) 16-mer,  $K_{16}^{app} = 7.8 \times 10^{22}$  (ml/mg)<sup>15</sup>.

form. However, the association constants are consistently lower than that of the phosphorylated form. Thus, it may be concluded that both the phosphorylated and dephosphorylated forms of PFK undergo the same modes of association, although the phosphorylated form apparently exhibits a greater tendency to form oligomers.

In analyzing the sedimentation results, it was assumed that there occurred no significant conformational changes during subunit assembly. In order to establish the validity of this assumption, CD was employed to monitor the secondary structure of PFK as a function of protein concentration ranging from 0.02 to 2.0 mg/ml. The results of this study are shown in fig. 3A. Within experimental uncertainties, there is no detectable difference in the far-ultraviolet CD spectra of PFK within the range of protein concentration studied. In addition, the same result was observed regardless of the state of phosphorylation of PFK, as shown in fig. 3A. Hence, it can be concluded that there is no significant change in the secondary structure of PFK monomer and other oligomeric species. Another structural investigation was conducted to probe the solvent accessibility of fluorophores and the results are shown in fig. 4. It is evident that the value of weight-average  $K_Q$  is protein concentration dependent. The weight-average  $K_Q$  increases from  $2.7 \pm 0.1$  to  $3.2 \pm 0.1$  M<sup>-1</sup> when the protein concentration changes from 18 to 1760 μg/ml. These results indicate an increase in accessibility

of the tryptophan residues upon oligomer formation. The increase in accessibility may simply be a reflection on the exposure of fluorophores to

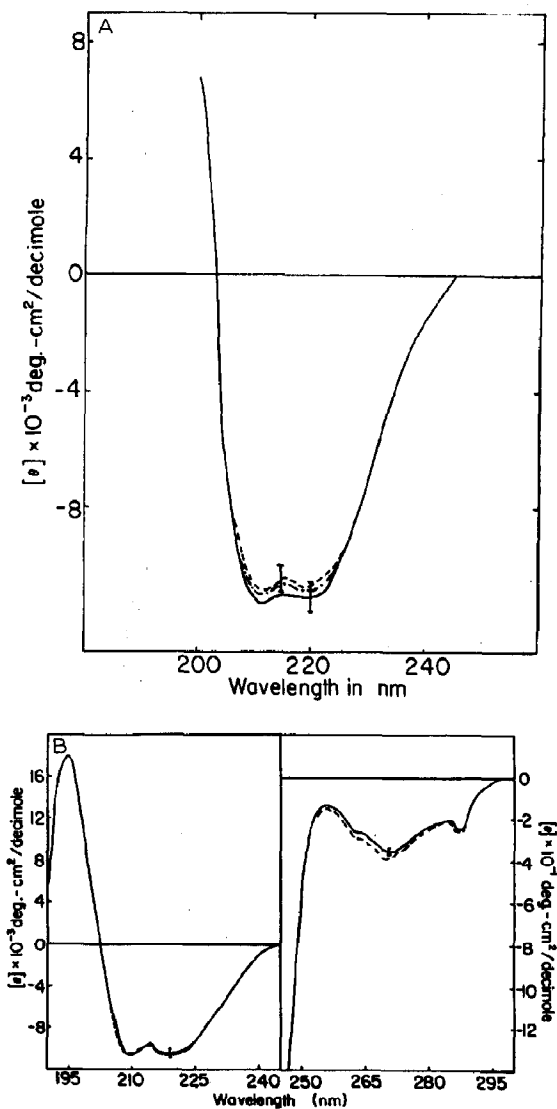


Fig. 3. CD spectra of PFK in TEMA buffer at pH 7.0 and 23°C. (A) Far-ultraviolet spectra of PFK as a function of protein concentration: protein concentrations (in μg/ml); (—) 0.02; (---) 0.2; (- - -) 2.0. Identical spectra were observed with either phosphorylated or dephosphorylated enzyme. (B) Far- and near-ultraviolet spectra of PFK with (—) and without (---) F6P. The protein concentration was 1.8 mg/ml in both cases. The bars represent the magnitudes of the maximum deviations.

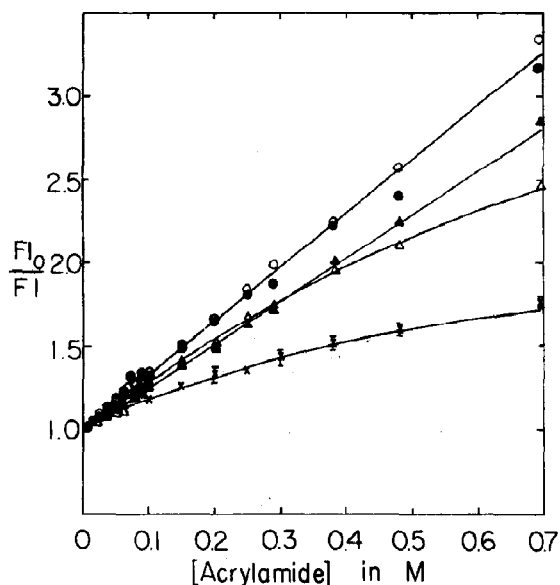


Fig. 4. Stern-Volmer plots of PFK. Protein concentrations (in  $\mu\text{g/ml}$ ): ( $\circ$ ) 1760, ( $\bullet$ ) 180, ( $\blacktriangle$ ) 115, ( $\triangle$ ) 18, ( $\times$ ) 20  $\mu\text{g/ml}$  in the presence of 1 mM F6P. The bars represent the maximum deviations of multiple data sets.

solvent or increase in dynamic motion of peptides containing these fluorophores when subunit-subunit interaction occurred. Since the reported value of  $K_Q$  is a weight-average, an alternative interpretation of the fluorescence data is that at higher concentration some fraction of the fluorophores is shielded, leaving the mostly exposed species still accessible to solvent. It is the dominating contribution of this fraction that leads to the apparent increase in weight-average  $K_Q$ . The fluorescence and CD data may still be consistent, since the former technique is much more sensitive to local environmental changes where CD yields information on the global structure. In summary, the assumption employed in the estimation of the values of  $S_i^\circ$  by eq. 4 is most likely valid, i.e., no significant structural changes take place among various oligomeric species of PFK.

In earlier studies from this laboratory, it was shown that the PFK tetramer assumes a  $S_{20,w}^\circ$  of 12.4 S in the presence of the substrate F6P instead of the 13.5 S form observed in its absence [2,3,27,34,35]. This change in the hydrodynamic properties of the PFK tetramer may be associated

with modifications in secondary/tertiary structures in PFK induced by substrate; hence, the effect of F6P on the structure of PFK was monitored by both CD and fluorescence quenching. The CD spectrum, as shown in fig. 3B, indicates no significant perturbation of the PFK structure in the presence of 0.2 mM F6P. The fluorescence quenching results (fig. 4) show that the weight-average  $K_Q$  assumes a lower value of  $1.6 \pm 0.1 \text{ M}^{-1}$  in the presence of 0.2 mM F6P, indicating that the substrate induces an apparent decrease in the accessibility of some fraction or all of the tryptophan residues to the solvent. The combination of the CD and fluorescence data implies that F6P does not induce significant changes in the secondary structure of PFK, although the local environments of the tryptophan residues might be perturbed. Thus, the observed change in hydrodynamic properties of PFK in the presence of F6P most likely is related to a rearrangement of subunit-subunit contacts without extensive secondary structural changes in the subunit.

The steady-state kinetic parameters of the various forms of PFK were monitored as a function of protein concentration using F6P as the variable substrate. The concentration of ATP was kept constant at 1 mM in TEMA buffer at pH 7.0 and 23°C. The results of these studies are listed in table 2. At 0.1  $\mu\text{g/ml}$ , the phosphorylated forms of PFK, be it in vivo or in vitro phosphorylated, exhibit a sigmoidal relationship between activity and F6P concentration. The apparent Michaelis constant,  $K_m$ , assumes a value of  $190 \pm 20 \mu\text{M}$ . Further analysis of the data in the form of a Hill plot [33] to assess the degree of cooperativity reveals that the Hill coefficient,  $n$ , is  $1.9 \pm 0.1$ . Repeating the same experiments with a PFK con-

Table 2

Summary of steady-state kinetic data for PFK and de-PFK

Preparation	[Protein] ( $\mu\text{g/ml}$ )	$K_m^{\text{app}}$ ( $\mu\text{M}$ )	$n$
Phosphorylated	0.1	$190 \pm 20$	$1.9 \pm 0.1$
	1.0	$88 \pm 5$	$1.3 \pm 0.1$
Dephosphorylated	0.1	$77 \pm 7$	$1.6 \pm 0.1$
	1.0	$50 \pm 3$	$1.1 \pm 0.1$



centration of 1.0  $\mu\text{g/ml}$  yielded results that are significantly different. The value of  $K_m$  increased approx. 2-fold with increased protein concentration. The Hill coefficient,  $n$ , also diminished, implying a decrease in the degree of cooperativity. A parallel steady-state kinetic study was conducted with dephosphorylated PFK. The same trend was observed, as summarized in table 2. The values of  $K_m$  and  $n$  decreased with increasing concentration. These observations suggest that self-assembly of PFK plays a significant role in the regulation of enzyme activity, although phosphorylation of the enzyme modifies this role.

#### 4. Discussion

The association-dissociation of rabbit muscle PFK has been the subject of intensive investigation [1–3,27,36–41]. These studies all agree that PFK undergoes self-association, and that this association-dissociation is affected by various metabolites; however, in some of the studies, contradiction between the data exists concerning the role of this self-association in the regulation of the enzyme. Recent studies from this laboratory have shown that (a) native PFK undergoes self-association in a rapid, dynamic equilibrium, (b) this association-dissociation is qualitatively influenced by metabolites, and (c) self-association is linked to the conformational state of the enzyme [3,27,34, 35]. Hence, based on thermodynamic principles, the self-association of PFK must play a role in its regulation. Since the ultimate goal of this series of studies is to correlate quantitatively the various mechanisms of regulation and enzymic activity, it is imperative that the quantification of these reaction steps yields valid thermodynamic parameters. In the earlier studies of PFK self-association from this laboratory, some basic assumptions were made without direct evidence; thus, it is one of the goals of the present study to test the validity of these assumptions.

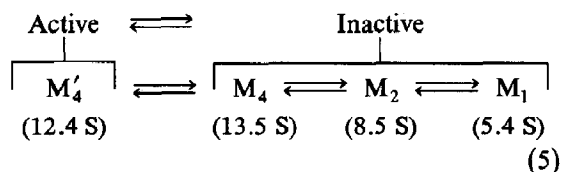
In the initial estimation of  $S^\circ$  for the various oligomeric forms of PFK, the assumption was made that no conformational changes occur during subunit assembly. The CD study shows that no significant secondary structural changes are

detectable as a function of protein concentration covering the same range as that involved in the sedimentation study. Hence, the initial assumption of no structural change is valid, and the values of  $S^\circ$  are  $S_1^\circ = 5.4$  S,  $S_2^\circ = 8.5$  S,  $S_4^\circ = 13.5$  S,  $S_8^\circ = 21.4$  S and  $S_{16}^\circ = 34.0$  S in the absence of substrate. Although the values employed in the earlier studies, namely,  $S_1^\circ = 4.95$  S,  $S_2^\circ = 7.6$  S,  $S_4^\circ = 13.5$  S,  $S_8^\circ = 19.7$  S and  $S_{16}^\circ = 34.0$  S, would yield better  $\sigma$  and SS values, there is no direct evidence to justify the adoption of those values. The lower  $S$  values were computed by using eq. 4 and  $S_4^\circ = 12.4$  S. Thus, one of us (J.C.L.) is withdrawing the other values used in the earlier studies in this laboratory. Nevertheless, a change in  $S^\circ$  values does not alter any of the conclusions on the mode of association as reported in those studies. Furthermore, the value of  $K_4$  is still within the experimental uncertainties ( $5.1 \times 10^5$  in ref. 27 and  $4.8 \pm 0.5 \times 10^5$  in this study), although the value of  $K_{16}$  is significantly different. Since most studies are focused on low protein concentrations ( $< 100$   $\mu\text{g/ml}$ ),  $K_4$  is much more pertinent than  $K_{16}$ .

Results from this laboratory also show that in the presence of substrate, PFK is active as a tetramer with  $S_4^\circ = 12.4$  S [2,3,27,35]. The CD study again shows that in the presence of a saturating amount of F6P, no detectable secondary structural changes occur. Thus, the difference between the inactive 13.5 S and active 12.4 S tetramers can be the result of a reorganization of the subunit-subunit interaction, e.g., conversion from a square planar to tetrahedral symmetry. Such a proposal is in total agreement with the reported structural changes in the bacterial enzyme observed by X-ray crystallography [8,9]. Furthermore, such reorganization of the subunit-subunit interaction has also been observed for *E. coli* aspartate transcarbamoylase and rabbit muscle glycogen phosphorylase during their activation processes [42–44]. Apparently, this type of mechanism for regulating enzyme activity may be quite general.

Having established the quaternary structure of the active and inactive forms of PFK, it is of interest to elaborate on the effects of metabolites on the subunit assembly of PFK. Reports in the literature show that in the presence of inhibitor or

under inhibitory conditions, such as low temperature, dissociation of PFK is favored and a significant amount of dimeric PFK can be detected [2,38]. On the other hand, in the presence of activator or under activating conditions, such as high pH, association of the enzyme is favored [2]. Thus, the current working hypothesis can be summarized as:



In this scheme, PFK exists in two species, an active and an inactive state, which are in rapid equilibrium. The higher aggregates of  $M_8$  and  $M_{16}$  are omitted, since their presence is only detected at an enzyme concentration so high that they do not occur in significant amounts under normal assay conditions. The active state is represented by the tetrameric PFK with a sedimentation coefficient of 12.4 S. The inactive state consists of a mixture of species including the tetrameric PFK with a sedimentation coefficient of 13.5 S and its corresponding dimeric and monomeric forms. Any factor that shifts this equilibrium to the left would behave as an activator, whereas one that perturbs the equilibrium towards the right would act as an inhibitor [2]. The effect of activators on the basic steady-state kinetic parameters can be predicted. In general, in the formulation of the Monod-Wyman-Changeux model [45], for sigmoidal kinetics, an activator would shift the curve of activity vs substrate concentration to the left, yielding a lower value for the  $K_m$  and Hill coefficient. A molecular mechanism which would lead to such an observation is that in the case when the amount of active tetramer,  $M'_4$ , present in solution is increased. PFK would exhibit a higher affinity for its substrate, as indicated by a lower value for the apparent  $K_m$ . Therefore, the greater the amount of  $M'_4$  present, the closer is the  $K_m$  value to reflecting the actual affinity of the active enzyme for F6P. This would also be reflected in the Hill coefficient which is linked to the ability to shift the inactive species to the active form [45]. Hence,

when more tetramer is present, the addition of F6P should not cause the equilibrium to be shifted significantly, thus leading to a lower Hill coefficient. The opposite phenomenon would be observed if the solution contains a greater amount of lower aggregates such as seen at low protein concentrations or in the presence of inhibitors. Under these conditions, the steady-state kinetic parameters will be characterized by high values for the  $K_m$  and Hill coefficient.

The kinetic results from this study are consistent with the prediction based on the working hypothesis, although at present, a direct quantitative correlation cannot be attempted due to the complexity of the system and the lack of quantitative data for some specific steps of the reaction. Nevertheless, the kinetic behaviors of the phosphorylated and dephosphorylated PFK forms are totally consistent with the subunit assembly data.

Having established a model for the regulatory mechanism of rabbit muscle PFK, it is of interest to compare and contrast current knowledge on the regulatory mechanisms of mammalian and bacterial PFK. There are a number of basic differences between the physical properties of these enzymes. They are very different in the size of their subunits [46] and the bacterial enzyme essentially exists as a stable tetramer [47], whereas the mammalian muscle enzyme undergoes association-dissociation readily. However, there are many common features in the regulatory mechanism. Garel and co-workers [47,48] have reported that *E. coli* PFK can undergo reversible denaturation. The pathway of reassociation can be expressed as

$$4U \rightarrow 4M_1 \rightarrow 2M_2 \rightarrow M_4 \quad (6)$$

where U and M represent the unfolded and native states, respectively, and  $M_i$  denotes the enzyme with  $i$  subunits. The presence of various oligomeric species is exactly analogous to that of the muscle enzyme. In a further study on reversible denaturation, Deville-Bonne et al. [48] showed that F6P binds to the tetramer and prevents it from dissociation, and that in the presence of inhibitor the dissociation of dimer to monomer is prevented. These results imply that the substrate favors the formation of the tetramer and that the inhibitor binds to the dimer. These observations are in total

agreement with the reported effects of ligands on the association-dissociation behavior of the muscle enzyme (for a review, see ref. 49). Thus, these enzymes can undergo essentially the same type of reaction, although the magnitudes of the various equilibrium constants governing these reactions are different.

In summary, subunit assembly is an integral component of the allosteric regulatory mechanism of rabbit muscle PFK. However, much is yet to be accomplished before a quantitatively consistent linkage can be established among the processes of subunit assembly, conformational change, ligand binding and catalytic efficiency.

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