# Self-Association of Rabbit Muscle Phosphofructokinase: Effects of Ligands†

Lyndal K. Hesterberg‡ and James C. Lee*

ABSTRACT: The effects of ligands on the self-association of rabbit muscle phosphofructokinase (PFK) were investigated by velocity sedimentation at pH 7.0 and 23 °C. The concentration dependence of the weight-average sedimentation coefficient was monitored in the presence of these ligands. The mode of association and equilibrium constants characterizing each association step were determined by theoretical fitting of the sedimentation data. The simplest mode of association for the PFK system is $M \rightleftharpoons M_2 \rightleftharpoons M_4 \rightleftharpoons M_{16}$. Ligands and temperature would perturb the various equilibrium constants without altering the mode of association. The apparent equilibrium constants for the formation of tetramer, $K_4^{app}$, are increased in the presence of 0.1 mM ATP and 1.0 mM fructose 6-phosphate. The value of the sedimentation coefficient for the tetramer, $s_4°$, that would best fit the data is 12.4 S instead of 13.5 S determined in the absence of substrates, thus implying a structural change in the tetramer induced by substrates. Only an insignificant amount of dimer is present under the experimental conditions. The presence of activators, ADP or phosphate, enhances the formation of tetramers, and $s_4°$ assumes a value of 13.5 S. Similar results are obtained with decreasing concentrations of proton. The presence of the inhibitor, citrate, however, favors the formation of dimers. The equilibrium constants determined as a function of ADP concentration were further analyzed by the linked-function theory derived by Wyman [Wyman, J. (1964) *Adv. Protein Chem. 19*, 224-285], leading to the conclusion that the formation of a tetramer involves the binding of two additional molecules of ADP per monomer. Similar analysis results in a conclusion that the formation of a dimer involves the binding of one additional molecule of citrate per phosphofructokinase subunit.

Rabbit muscle phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) (PFK)[1] has been shown to be an important glycolytic enzyme which is subjected to allosteric regulation. It has been demonstrated that a variety of ligands can affect the kinetic activity of PFK (Goldhammer & Paradies, 1979). These ligands include substrates and products of the reaction, $H^+$, divalent cations, and intermediary metabolites such as citrate, 2,3-diphosphoglycerate, and creatine phosphate. The mechanism by which these ligands exert their allosteric effects has been the subject of intensive investigation.

Frieden and co-workers (Bock & Frieden, 1976a,b; Frieden et al., 1976; Pettigrew & Frieden, 1979a,b) have proposed that ligands exert their influence by binding preferentially to either protonated (inactive) or unpronotated (active) forms of the enzyme, thus shifting the apparent p$K$ of an important ionizable group and leading to the inactivation of the enzyme. In this context, ATP is proposed to bind to inactive forms, while F6P would bind to the active forms. Activators of PFK, such as ADP, are proposed to exert their effect by shifting the ratio of protonated to unprotonated forms of the enzyme. Inhibitors, such as citrate, however, do not directly alter this ratio, but rather, their effects are explained on the basis of induced differences in F6P binding.

However, PFK is also capable of undergoing self-association (Parmeggiani et al., 1966; Ling et al., 1965; Leonard & Walker, 1972; Aaronson & Frieden, 1972) in a rapid, dynamic equilibrium within the limits of resolution by sedimentation velocity experiments (Hesterberg & Lee, 1981). It has been clearly demonstrated qualitatively that the self-association equilibrium can be affected by the presence of ligands (Parmeggiani et al., 1966; Lad et al., 1973; Aaronson & Frieden, 1972; Hill & Hammes, 1975; Reinhart & Lardy, 1980). In general, substrates and activators were reported to favor PFK self-association, while inhibitors enhance the formation of smaller aggregates. There is, however, no quantitative analysis of the effects of ligands on the equilibrium. Furthermore, the role of self-association on the regulation of PFK activity is still unknown, although in the model proposed by Frieden self-association is not considered to play any role in enzyme regulation due to the apparently slow rate constant governing the association of subunits.

In an effort to gain more insights into the regulation of PFK, active enzyme sedimentation analysis of PFK has demonstrated that the only detectable active form is the tetramer (Hesterberg & Lee, 1979, 1980). The combined observations that (1) the subunits of PFK are in a rapid, dynamic equilibrium and that (2) the smallest observable active form is the tetramer lead to the following questions: Are aggregates smaller than the tetramer inactive? Has the equilibrium of self-association been perturbed by the presence of substrates so much so that the tetramer is the predominant species?

By combining the knowledge on the mass distribution of PFK between the various polymeric forms under a defined set of conditions and the enzymatic activity monitored under exactly the same conditions, one may then determine if changes in the mass distribution of PFK correlate with those in the enzymatic activity, assuming the tetramer is the smallest active form and all smaller oligomers are inactive. If the activity does change as a function of the mass distribution of the

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‡ Present address: Max-Planck-Institute for Biophysical Chemistry, D-3400 Goettingen, Federal Republic of Germany.

[1] Abbreviations: PFK, phosphofructokinase; AEC, active enzyme centrifugation; F6P, fructose 6-phosphate; AMP-PNP, adenylyl imidodiphosphate; TEMA buffer, 25 mM Tris–$CO_3$ with 1 mM EDTA, 6 mM $MgCl_2$, and 3.4 mM $(NH_4)_2SO_4$ buffer at pH 7.0; GEMA buffer, 25 mM glycylglycine buffer containing 1 mM EDTA, 6 mM $MgCl_2$, and 3 mM $(NH_4)_2SO_4$ at pH 8.55.

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enzyme, then the results would suggest that PFK activity must, to some degree, be regulated through the change in its quaternary structure.

Reports in the literature do not provide quantitative information to test this possibility. There are no reports on the mode of association or on the effects of ligands on the association equilibrium. Recent advances in theoretical simulation of sedimentation velocity patterns have allowed quantitative analysis of the self-association of PFK in the absence of ligands (Hesterberg & Lee, 1981). Results indicate that the association equilibrium at pH 7.00 and 23 °C can best be described as $M \rightleftharpoons M_4 \rightleftharpoons M_{16}$ with $K_4^{app} = 5.06 \times 10^5$ (mL/mg)$^3$ and $K_{16}^{app} = 3.25 \times 10^{23}$ (mL/mg)$^{15}$.

By use of the same techniques, this paper reports results of a detailed study on the association equilibrium of PFK in the presence of allosteric effectors, substrates, $H^+$, and phosphate.

## Materials and Methods

ATP, fructose 6-phosphate, citrate, and ADP were purchased from Sigma Chemical Co. AMP-PNP was obtained from Boehringer Mannheim. These were used without further purification.

Phosphofructokinase was purified, stored, and assayed as described previously (Hesterberg & Lee, 1981). The specific activity was ≥300 units/mg. The enzyme was equilibrated in the appropriate buffer and the concentration determined by the absorbance at 280 nm with an absorptivity of 1.07 L/(g·cm) (Hesterberg & Lee, 1981).

Sedimentation velocity studies were carried out and analyzed with previously published procedures (Hesterberg & Lee, 1981). Weight-average sedimentation coefficients, $\bar{s}$, were determined from the centroid, a close approximation of the second moment, of the scanner-traced boundaries. The observed weight-average sedimentation coefficients were normalized to standard conditions by correcting for solvent density and viscosity.

## Results

One of the basic requirements of successful analysis of the sedimentation velocity data is that the association must exist in a rapid, dynamic equilibrium. This has been observed for the enzyme in the absence of ligand (Hesterberg & Lee, 1981). However, since the PFK–ligand interactions will be thermodynamically coupled to the self-association equilibrium, studies must therefore be conducted to analyze the self-association equilibrium in the presence of each of the ligands. The three criteria utilized previously (Hesterberg & Lee, 1981) were applied, and in each case the results indicate that the system is indeed in a rapid, dynamic equilibrium. These criteria include the following: (1) the value of $\bar{s}_{20,w}$ is not a function of $\omega^2$; (2) measuring $\bar{s}_{20,w}$ as a function of time for samples diluted from a stock solution revealed no time dependence of the value for $\bar{s}_{20,w}$; and (3) analyzing the derivative tracings as a function of protein concentration qualitatively follows the patterns predicted by Gilbert (1963) for a self-associating system in a rapid, dynamic equilibrium. To ensure that the data are amenable to such an analysis, the ratio of the concentration of ligand to PFK monomer was maintained at greater than 30-fold under all experimental conditions so as to circumvent the potential artifact of ligand-mediated association as shown by Cann (1970).

*Effects of Substrates.* One of the primary interests of this study is the effect of substrate(s) on the self-association of PFK. Results of AEC studies in GEMA and TEMA buffers have shown that the only observable active form of PFK between 0.3 and 25 μg/mL sediments as a tetramer with a value

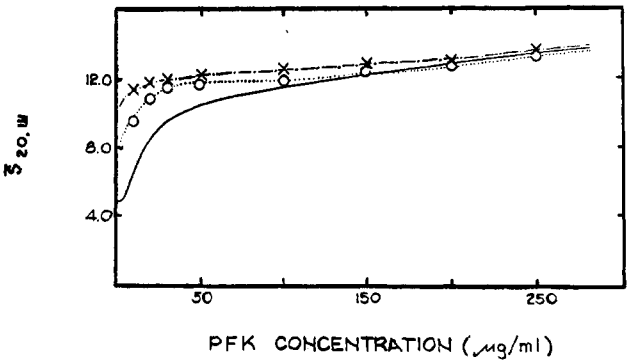


FIGURE 1: Effect of substrate on the concentration dependence of the weight-average sedimentation coefficient, $\bar{s}_{20,w}$, of PFK in TEMA buffer at pH 7.00, 23 °C. Symbols and the theoretical best fit are the following: 1.0 mM fructose 6-phosphate (·····), $M \rightleftharpoons M_4 \rightleftharpoons M_{16}$ with $s_4^\circ = 12.4$ S and (×) representing the average of multiple data sets; 0.1 mM ATP (···) $M \rightleftharpoons M_4 \rightleftharpoons M_{16}$ with $s_4^\circ = 12.4$ S (○) representing the average of multiple data sets. The solid line represents the concentration dependence of $\bar{s}_{20,w}$ of PFK in TEMA buffer at pH 7.00, 23 °C.

of $\bar{s}_{20,w} = 12.4 \pm 0.5$ S (Hesterberg & Lee, 1980; Hesterberg, 1980; Hesterberg et al., 1981). However, the concentration dependence of the sedimentation profile of PFK in the absence of ligands reveals that at PFK concentrations below 50 μg/mL substantial amounts of tetramer and monomer both exist (Hesterberg & Lee, 1981). At protein concentrations below 2 μg/mL, essentially all of the enzyme exists as monomer. It poses the following question: Can the substrates induce a shift in the association equilibrium to account for these observations? It is not possible to study PFK self-association with boundary sedimentation velocity in the presence of both fructose 6-phosphate and ATP without having the complicating effects of products present also. Therefore, the concentration dependence of the sedimentation coefficient was determined for PFK in the presence of either 0.1 mM ATP or 1 mM F6P. The high absorptivity of ATP limits the concentration of nucleotide employed in this study. Concentrations of nucleotide higher than 0.1 mM provide too great of a background absorbance for accurate tracings by the scanner. The effect of 0.1 mM ATP on the concentration dependence of $\bar{s}_{20,w}$ of PFK is shown in Figure 1. The formation of PFK tetramers is favored. The effect of 1.0 mM F6P is also shown in Figure 1. A similar enhancement is observed. It is evident that the effects of the substrates are observable most readily at lower PFK concentrations, and no significant effect is detected at protein concentrations above 300 μg/mL under the experimental conditions. This observation implies that the primary effect of the substrates is expressed in the initial tetramerization of the enzyme with little or no detectable influence on the formation of higher aggregates within the limited range of protein concentration studied.

Table I shows the results of the theoretical calculation and curve fitting of the data. The simplest model which fits the sedimentation velocity data the best for either substrate yields a stoichiometry of $M \rightleftharpoons M_4 \rightleftharpoons M_{16}$ and values of $7.56 \times 10^9$ and $3.97 \times 10^7$ (mL/mg)$^3$ for $K_4^{app}$ in the presence of F6P and ATP, respectively. Obviously, either substrate is capable of inducing a large shift in the equilibrium and favors the formation of tetrameric PFK.

It is conceivable that the presence of both ATP and F6P may lead to a greater effect in the equilibrium than the summation of either F6P or ATP alone could. In an effort to more closely examine this possibility, an ATP analogue, AMP-PNP, was used. This analogue is structurally similar to ATP (Yount et al., 1971a,b) but is not a substrate of the enzyme and is not

Table I: Summary of Ligand Effects

| ligands[a] | $s_4$ | stoichiometry | $K_2^{app}$ (mL/mg) | $K_4^{app}$ (mL/mg)$^3$ | $K_{16}^{app}$ (mL/mg)$^{15}$ | $\sigma$ |
|---|---|---|---|---|---|---|
| none | 13.5 | 1-4-16 | | $5.06 \times 10^5$ | $3.25 \times 10^{23}$ | |
| 1.0 mM F6P | 12.4 | 1-4-16 | | $7.56 \times 10^9$ | $1.49 \times 10^{40}$ | 0.19 |
| | 13.5 | | | | | 1.09 |
| 0.1 mM ATP | 12.4 | 1-4-16 | | $3.97 \times 10^7$ | $9.92 \times 10^{30}$ | 0.29 |
| | 13.5 | | | | | 0.96 |
| 0.1 mM AMP-PNP + 1.0 mM F6P[b] | 12.4 | 1-4-16 | | $3.00 \times 10^{11}$ | $1.50 \times 10^{46}$ | |
| $2.82 \times 10^{-9}$ M $H^+$ (pH 8.55) | 13.5 | 1-4-16 | | $1.15 \times 10^7$ | $4.6 \times 10^{28}$ | |
| 0.1 M phosphate | 13.5 | 1-4-16 | | $6.53 \times 10^6$ | $3.29 \times 10^{27}$ | |
| 10 mM citrate | 13.5 | 1-2-4-16 | 443.75 | $5.00 \times 10^5$ | $4.29 \times 10^{21}$ | |
| 1.0 mM citrate | 13.5 | 1-2-4-16 | 175.14 | $5.65 \times 10^5$ | $5.47 \times 10^{21}$ | |
| 0.5 mM citrate | 13.5 | 1-2-4-16 | 69.10 | $4.18 \times 10^5$ | $5.31 \times 10^{22}$ | |
| 0.1 mM citrate | 13.5 | 1-2-4-16 | 5.96 | $4.56 \times 10^5$ | $1.01 \times 10^{23}$ | |
| 0.10 mM ADP | 13.5 | 1-4-16 | | $6.20 \times 10^8$ | $3.99 \times 10^{35}$ | |
| 0.05 mM ADP | 13.5 | 1-4-16 | | $5.44 \times 10^7$ | $1.96 \times 10^{31}$ | |
| 0.01 mM ADP | 13.5 | 1-4-16 | | $6.94 \times 10^6$ | $6.48 \times 10^{27}$ | |

[a] All experiments were conducted in TEMA buffer at pH 7.00 at 23 °C. GEMA buffer was used at pH 8.55. [b] The equilibrium constants represent the minimum values possible.

hydrolyzed by the ATPase activity of the enzyme (Uyeda, 1970; Colombo et al., 1975). AMP-PNP appears to bind in a similar fashion as ATP and exhibits similar allosteric behavior by binding to both the catalytic and the regulatory sites (Hofer & Pette, 1968; Wolfman et al., 1978). Control studies revealed no detectable hydrolysis of AMP-PNP for periods up to 24 h under the present experimental conditions. Furthermore, the sedimentation behavior of PFK in the presence of 0.1 mM AMP-PNP is identical with that of ATP.[2] It may therefore be concluded that AMP-PNP exerts a similar effect on PFK as ATP. The concentration dependence of the self-association of PFK in the presence of 1 mM F6P and 0.1 mM AMP-PNP was studied. The self-association is enhanced at PFK concentrations below 200 μg/mL with no significant changes at the higher protein concentrations studied. The observed value for $\bar{s}_{20,w}$ is 12.4 S at 10 μg/mL PFK, indicating that PFK does not undergo detectable dissociation even at such a low concentration, although dissociation of the tetramer can be easily detected with either substrate alone under these conditions.

The strong association complicates the theoretical calculation and curve fitting, as the dissociation of tetramer was not observed. One may calculate only for the minimum apparent equilibrium constants by assuming that PFK will undergo dissociation at concentrations below 10 μg/mL. The result of such a calculation yielded a *minimum* value for $K_4^{app}$ of $3.0 \times 10^{11}$ (mL/mg)$^3$ with $s_4^\circ$ = 12.4 S.

*Effects of Activators.* The activation of PFK activity by ADP, especially in the presence of high levels of ATP or citrate, has been widely reported (Uyeda, 1979; Passonneau & Lowry, 1963; Mansour, 1972; Goldhammer & Paradies, 1979). If the tetrameric form of the enzyme is the only active species, and the activity is modulated through the self-association equilibrium, then one might expect an "activator" of the enzyme to enhance the tetrameric form of the enzyme. If, however, the activation is strictly on a kinetic level, then one may not anticipate observing any effect of the ligand on the quaternary structure of PFK.

The effect of ADP on the concentration dependence of the self-association of PFK is shown in Figure 2. In the presence of 0.10 mM ADP, the formation of the tetrameric form of PFK is favored. The concentration dependence of PFK in the presence of ADP is significantly different from that of PFK alone. The effect, however, is only observed at enzyme concentrations below 300 μg/mL. At higher concentrations, no significant effects of ADP can be observed under the present experimental conditions.

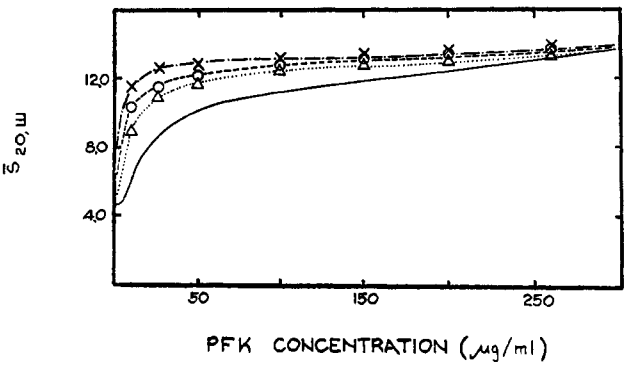


FIGURE 2: Effect of ADP on the concentration dependence of the weight-average sedimentation coefficient, $\bar{s}_{20,w}$, of PFK in TEMA buffer at pH 7.00, 23 °C. Symbols corresponding to the concentration (mM) of ADP are the following: (Δ) 0.01; (O) 0.05; (×) 0.10. The solid line represents the concentration dependence of $\bar{s}_{20,w}$ of PFK in TEMA buffer at pH 7.00, 23 °C, and the broken lines represent the theoretical fit of the experimental data by using the association model of $M \rightleftharpoons M_4 \rightleftharpoons M_{16}$ and $s_4^\circ$ = 13.5 S. The data points are the average of multiple data sets.

In an effort to delineate the effect of ADP, the association of PFK was studied as a function of ADP concentration, and the results are shown in Figure 2. Theoretical calculation and curve fitting of the data reveal that the simplest mode of self-association is $M \rightleftharpoons M_4 \rightleftharpoons M_{16}$ with $s_4^\circ$ = 13.5 S in all cases. However, the value for $K_4^{app}$ is $6.20 \times 10^8$, $5.44 \times 10^7$, and $6.94 \times 10^6$ (mL/mg)$^3$ in the presence of 0.10, 0.05, and 0.01 mM ADP, respectively, as shown in Table I. These values of $K_4^{app}$ represent 14–1225-fold over that in the absence of ADP. It is evident that the significant effect of ADP is to enhance the formation of PFK tetramers under the experimental conditions.

Phosphate has been reported to be an activator (Goldhammer & Paradies, 1979), and it is also included in the buffer systems of a large number of studies (Pavelich & Hammes, 1973; Parr & Hammes, 1975, 1976; Bock & Frieden, 1974; Ling et al., 1965), possibly due to the stabilizing effect of phosphate on the enzyme (Lad et al., 1973). The effect of 0.1 M phosphate on the self-association of PFK was therefore studied at pH 7.00 and 23 °C. The results from theoretical calculations and curve fitting are summarized in Table I. The primary effect of phosphate is evidently on the initial tetram-

[2] M. A. Luther and J. C. Lee, unpublished experiments.

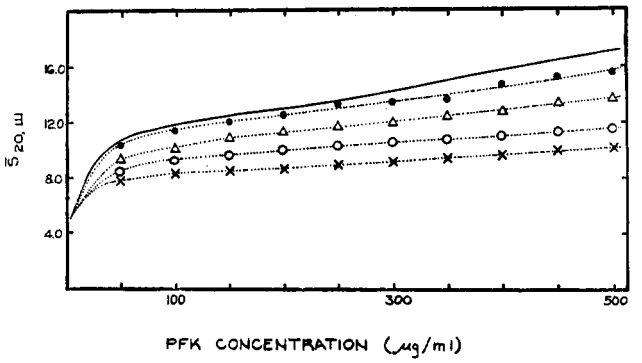


FIGURE 3: Effect of citrate on the concentration dependence of the weight-average sedimentation coefficient, $\bar{s}_{20,w}$, of PFK in TEMA buffer at pH 7.00, 23 °C. Symbols corresponding to the concentration (mM) of citrate are the following: (●) 0.1; (△) 0.5; (○) 1.0; (×) 10. The solid line represents the concentration dependence of $\bar{s}_{20,w}$ of PFK in TEMA buffer at pH 7.00, 23 °C; the broken lines represent the theoretical fit of experimental data by using the association model of $M \rightleftharpoons M_2 \rightleftharpoons M_4 \rightleftharpoons M_{16}$ and $s_4° = 13.5$ S. The data points represent the average of multiple data sets.

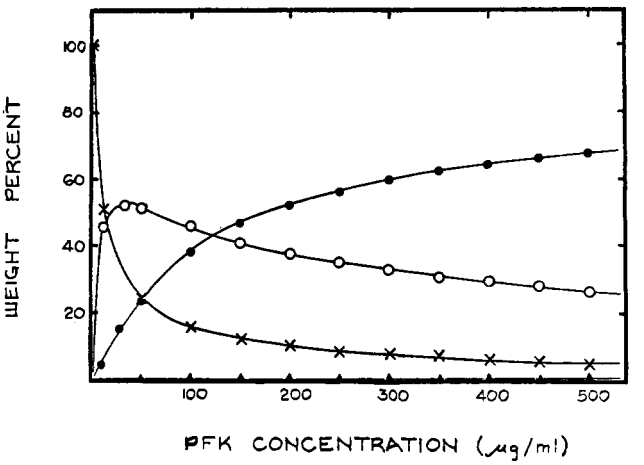


FIGURE 4: Mass distribution of PFK among polymeric forms for a self-association of $M \rightleftharpoons M_2 \rightleftharpoons M_4 \rightleftharpoons M_{16}$ as a function of protein concentration in the presence of 10 mM citrate. Symbols and apparent equilibrium constants are as follows: (×) monomer; (●) dimer, $K_2^{app} = 443.75$ mL/mg; (○) tetramer, $K_4^{app} = 5.00 \times 10^5$ (mL/mg)$^3$; (▲) 16-mer, $K_{16}^{app} = 4.29 \times 10^{21}$ (mL/mg).[15]

erization of PFK since the value of $K_4^{app}$ has increased about 13-fold.

Increasing the ionic strength of Tris–$CO_3$ buffer to a level comparable with that of 0.1 M phosphate has no detectable effect on the self-association of PFK. The effect observed for phosphate is therefore probably a reflection on a specific ligand effect rather than on a nonspecific ionic strength effect.

*Effect of an Inhibitor.* Citrate, a potent inhibitor of rabbit muscle PFK, is chosen as the ligand to study the effects of inhibitor on PFK self-association. The concentration dependence of the sedimentation behavior of PFK in the presence of citrate is shown in Figure 3. Increasing concentration of citrate induces a greater decrease in the observed values of the sedimentation coefficient, indicating a shift in the association–dissociation equilibrium toward the formation of smaller species. The stoichiometry of association which gives rise to the best fit is $M \rightleftharpoons M_2 \rightleftharpoons M_4 \rightleftharpoons M_{16}$. The major effect of citrate is apparently on $K_2^{app}$. The value of $K_2^{app}$ increases with increasing concentration of citrate, as shown in Table I. The value of $K_4^{app}$, however, is neither consistently nor significantly altered upon the addition of citrate, although the value of $K_{16}^{app}$ does significantly decrease with higher concentrations of citrate. These results indicate that two effects are apparently occurring upon the binding of citrate to PFK. The formation of dimer is favored in the presence of citrate with a concomitant dissociation of the 16-mer whereas there is no detectable influence on the formation of tetramer. The mass distribution of PFK among polymeric forms as a function of protein concentration is shown in Figure 4. It is evident that the concentration of dimer increases significantly with total protein concentration between 0 and 50 μg/mL.

*Effect of Hydrogen Ion.* The kinetic properties and the self-association of PFK are closely related to the concentration of hydrogen ion. The concentration dependence of $\bar{s}_{20,w}$ was monitored at pH 8.55 to understand more fully the effect of pH on the self-association of PFK. Exposure of PFK to the higher pH resulted in a greater increase in $\bar{s}_{20,w}$ with increasing protein concentration, indicating that at higher pH the equilibrium is shifted in favor of the formation of PFK tetramers. Curve fitting of the data reveals that the best fit is obtained with a stoichiometry of $M \rightleftharpoons M_4 \rightleftharpoons M_{16}$ with $s_4° = 13.5$ S, as shown in Table I. Models including significant amounts of other intermediate species such as $M_2$ or $M_8$ do not fit the data. Thus, the mode of association remains unaltered between pH 7.00 and 8.55, but increasing concentrations of $H^+$ appear to favor dissociation of PFK.

## Discussion

The effects of ligands on the self-association of PFK have been the subjects of previous investigations; however, results of this study constitute the initial quantitative analysis including determination of the mode of association and the equilibrium constants that govern the reactions.

On the basis of the results obtained in this study, the *simplest* general model of self-association for the PFK system is $M \rightleftharpoons M_2 \rightleftharpoons M_4 \rightleftharpoons M_{16}$. The presence of significant experimental uncertainties does not enable one to completely exclude the presence of other species. However, the present model represents a system that contains the least number of detectable species whose behavior is consistent with the experimental data. The presence of ligands perturbs the various equilibrium constants without altering the mode of association. Substrates and activators would enhance the formation of PFK tetramers, and the amount of dimers present is insignificant under the conditions examined. Citrate and low temperature (Hesterberg & Lee, 1981), however, favor the formation of dimers at the expense of the other species.

Both substrates enhance the self-association of PFK. However, the value of $s_4°$ that would lead to the best fit of the data is 12.4 S in both cases. The value for $\sigma$ is much better than that obtained by using $s_4° = 13.5$ S. The value of 12.4 S required for the quantitative analysis of the sedimentation velocity data is in very good agreement with the results of the AEC studies, which also observed the active tetramer to sediment at 12.4 S. This excellent agreement between the two techniques may be coincidental, as the combined effects of the experimental errors and assumptions inherent in both approaches on the reported results cannot be predicted. However, as both approaches indicate a decrease in the sedimentation coefficient of PFK in the presence of substrates, it is reasonable to assume that it is real. The analytical methods employed in these studies are not sufficiently accurate to define the precise extent of decrease in the sedimentation coefficient but most likely indicate that a conformational change in the enzyme is occurring upon the binding of substrate. Such conformational changes have been observed previously in other enzyme systems. Smith & Schachman (1973) observed an increase in the sedimentation rate of glyceraldehyde phosphate dehydrogenase upon the binding of substrate. Howlett &

Schachman (1977) observed a decrease in the sedimentation coefficient of aspartate transcarbamylase upon the binding of substrate. Thus, a change in the intrinsic sedimentation coefficient of PFK upon the binding of substrate is not without precedent.

An apparent decrease in the sedimentation coefficient may be observed as a consequence of an artifact in a ligand-mediated associating system (Cann & Goad, 1972; Cann, 1970). Such behavior has been demonstrated conclusively in the vinblastine-induced self-association of brain tubulin (Weisenberg & Timasheff, 1970; Lee et al., 1975; Na & Timasheff, 1980a,b). The conditions which lead to such a decrease in the observed sedimentation coefficient generally require a strong interaction between protein and ligand at a low ratio of protein concentration to that of the ligand so that a stable gradient of ligand is generated across the sedimenting boundary in the centrifuge cell. In this study, the ratio of the concentration of substrate to PFK monomer is at least 30-fold. The large excess of ligand over protein precludes the formation of a stable ligand gradient; thus, the observed change in the sedimentation coefficient is probably a reflection on the change in the hydrodynamic properties of the PFK tetramer.

The effects exerted by substrates on the association equilibrium of PFK suggest that the polymeric state of the enzyme and thus the activity of PFK may be modulated to some extent through the association–dissociation equilibrium.

In order to further examine this possibility, the effects of a kinetic activator and inactivator were observed. If the tetramer is the smallest active form of PFK, one would predict that an activator would enhance PFK association to the tetramer, assuming the effect is mediated through the association–dissociation equilibrium. In a corresponding manner, an inactivator would be predicted to shift the equilibrium in favor of the smaller, inactive forms of PFK. The ligand could facilitate the association–dissociation by binding preferentially to one form of the enzyme. In order to test this possibility, the interactions between PFK and an activator, ADP, and an inactivator, citrate, were analyzed.

The observed values of $K_4^{app}$ in the presence of ADP increases with increasing ADP concentrations. Although the result substantiates the effect on the association of PFK, the eqiulibrium constants are a reflection of the thermodynamic linkage of ADP binding and PFK self-association. A quantitative analysis of the ligand–PFK interaction is possible by using the linked-function theory (Wyman, 1964).

In the general case, as outlined by Na & Timasheff (1980b), the equilibrium constant $k$ can be expressed as $k = [C]/([A][B])$ where [A], [B], and [C] are the molar concentrations of two reactants which give rise to product, also in terms of molar concentration. When this equilibrium is affected by a ligand, X, the thermodynamic linkage between $k$ and the interaction with the ligand are given by (Wyman, 1964)

$$\left(\frac{\partial \ln k}{\partial \ln a_X}\right)_{T,P,a_{j\neq X}} = \left(\frac{\partial m_X}{\partial m_C}\right)_{T,P,m_{j\neq C}} - \left(\frac{\partial m_X}{\partial m_B}\right)_{T,P,m_{j\neq B}} \quad (1)$$

where $a_X$ is the thermodynamic activity of ligand X and $(\partial m_X/\partial m_i)_{T,P,a_{j\neq X}}$ is the preferential binding of the ligand X to species i of the macromolecule. This is only valid when the activities of all other ligands are held constant (Aune & Timasheff, 1971). When the ligand X interacts so strongly with the macromolecule that the free ligand concentration is much less than the molar concentration of water, the preferential binding term actually reduces to the following equation:

$$\frac{\partial \ln k}{\partial \ln a_X} = \frac{\partial \ln k}{\partial \ln X} = \bar{X}_C - \bar{X}_A - \bar{X}_B \quad (2)$$

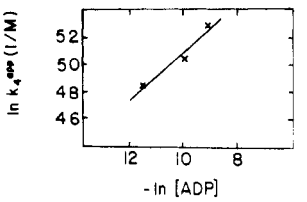


FIGURE 5: Plot of ln $k_4^{app}$ as a function of ln [ADP]. The line is a least-squares fit of the data.

The replacement of $a_X$ with X is possible since the activity coefficient under these conditions is approximately equal to 1.00. The reaction equilibrium constant in eq 2 is an apparent quantity, since there is no way to distinguish between molecules with different extents of binding of the ligand X. $\bar{X}_i$ in eq 2 is defined as the average number of moles of ligand X bound per mole of macromolecule j. $\bar{X}_C - \bar{X}_A - \bar{X}_B = \Delta\bar{X}$, which is defined as the difference between the average ligand binding of the products and the reactants. $\Delta\bar{X}$ will have a value somewhere between zero and the stoichiometry of ligand binding. An analysis of $\partial \ln k/(\partial \ln [X])$ as a function of the ligand concentration should give rise to the ligand stoichiometry of the reaction. Thus, for the case in hand, $\Delta\bar{X}$ is the slope of the graph of ln $k_4^{app}$ (molar) as a function of ADP concentration, where $k_4^{app} = (M_1^3/4)K_4^{app}$ and $M_1$ is the molecular weight of PFK monomers at 83 000. Figure 5 shows the linear plot of ln $k_4^{app}$ vs. ln [ADP]. The slope, $\Delta\bar{X}$, equals 1.9. The simplest interpretation of this result is that about two more ADP's are bound per subunit when it is in the tetrameric state than when it exists in the monomeric state. This can be tentatively interpreted as ADP binding to both the catalytic and the regulatory site of a PFK subunit in a tetrameric state, while no ADP is bound to the dissociated subunit. However, the slope obtained from this may be overstated, for the concentration term is defined as the free molar concentration of ADP. In the present calculations, it was assumed that the amount of bound ADP is negligible when compared to the total concentration of ADP. As no direct binding data for ADP have been reported, the $K_m$ value of 30 $\mu$M for ADP will be used (Hanson et al., 1973) to evaluate the validity of the assumption. Calculations reveal that below 200 $\mu$g/mL PFK concentrations the bound/free ratio of ADP is negligible. Within this range, the assumption is valid, and it is within this range that the greatest effects are observed on the $\bar{s}_{20,w}$ of PFK. At protein concentrations above 200 $\mu$g/mL, the amount of ADP bound increases until the bound/free ratio no longer is insignificant. If $\Delta\bar{X}$ is recalculated by using the concentration of free ADP present at an enzyme concentration of 500 $\mu$g/mL, the greatest error possible due to this assumption can be determined. $\Delta\bar{X}$ determined under this set of conditions decreases to 1.70. This represents the maximum error possible, although no ADP-induced effects are observed above 200 $\mu$g/mL. Therefore, the assumption appears to be valid for the analysis of PFK–ADP interactions.

The binding of the kinetic inactivator citrate to PFK results in significant changes in the association equilibrium. With increasing concentrations of citrate, the association equilibrium shifts to favor smaller aggregates of PFK. In a manner analogous to the effect of lower temperatures (Hesterberg, 1980; Hesterberg & Lee, 1981), citrate significantly enhances the dimerization of PFK with little effect on $K_4^{app}$. As was the case with ADP, the binding of citrate and the self-association of PFK are thermodynamically linked, and the sedimentation data for PFK in the presence of citrate can be analyzed. The slope of the plot of ln $k_2^{app}$ vs. ln [citrate] (Figure 6) equals $\Delta\bar{X}$, which has a value of 1.1. Under all the

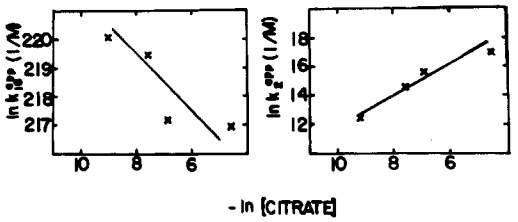


FIGURE 6: (Right panel) Plot of ln $k_2^{app}$ as function of ln [citrate]. (Left panel) Plot of ln $k_{16}^{app}$ as function of ln [citrate]. The lines are least-squares fits of the data.

conditions of these studies, the total citrate concentration is a good approximation for the free molar concentration of citrate, as Colombo et al. (1975) indicated that the binding constant for citrate is weak with $K_D = 5 \times 10^{-4}$ M. The effect of any error in this approximation would be to lower the value of $\Delta\bar{X}$ observed. Thus, it appears that citrate binds preferentially to dimeric PFK. As mentioned earlier, $K_{16}^{app}$ for the self-association of PFK decreases with increasing concentrations of citrate. The slope from the plot of ln $k_{16}^{app}$ vs. ln [citrate] (Figure 6) yields a value of −1.0 for $\Delta\bar{X}$. This result can be tentatively interpreted as that upon the formation of a 16-mer the monomeric subunits must lose, on the average, one citrate molecule per subunit. The equilibrium for protein self-association in the presence of citrate must shift in favor of those polymeric species which can bind the greatest average number of citrate molecules per subunit, namely, dimer, and away from those polymeric species which bind the least average number of citrates, i.e., 16-mer.

The effect of $H^+$ concentration on both PFK aggregation and kinetic activity has been demonstrated frequently. All of the studies agree that lower $H^+$ concentrations favor association of the enzyme with diminished allosteric effects. Conversely, higher $H^+$ concentrations favor dissociation of the enzyme and enhanced allosteric effects. This study is the first quantitative analysis of the effect of $H^+$ concentration on the self-association equilibrium of PFK.

At pH 8.55, the association to tetramer at low PFK concentrations is strongly enhanced. The value of $K_4^{app}$ at pH 8.55 is over 20-fold greater than that at pH 7.00. If the activity of PFK is regulated through changes in the association–dissociation equilibrium, then one would predict that allosteric effectors would have diminished effects at pH 8.55 when compared to those at pH 7.00. This is intuitively apparent since any allosteric effects must be balanced thermodynamically with the much larger $K_4^{app}$ at pH 8.55 and explains the results of previous kinetic studies (Uyeda, 1979; Goldhammer & Paradies, 1979), i.e., a decrease in the observed allosteric effect at high pH.

The results of these studies cannot provide a mechanism for the observed changes in the association equilibrium of PFK, but they do reveal the necessity of taking the polymerization state of the enzyme into account when analyzing PFK data at various pHs.

Results of this study reveal a strong influence of phosphate on the self-association of PFK by increasing the association constant 10-fold. Knowing that the equilibrium of the enzyme system is altered by the presence of phosphate, the question must arise as to whether or not the kinetic properties of the enzyme are also altered. If the presence of phosphate enhances PFK tetramerization and this change in the physical state is related to the enzymatic activity, one might predict, under a defined set of conditions, that the specific activity of PFK as a function of protein concentration would reveal sigmoidal kinetics in the absence of phosphate, while exhibiting hyperbolic kinetics in the presence of it.

Kinetic studies by Hofer & Pette (1968) have shown that the relative specific activity of PFK as a function of PFK concentration at pH 7.1 reveals sigmoidal (cooperative) kinetics in 0.1 M glycylglycine buffer but exhibits hyperbolic kinetics in 0.1 M phosphate buffer. The increase in $K_4^{app}$ observed in this study is sufficient to suggest an explanation for the findings of Hofer and Pette. The mass distribution of PFK in 0.1 M glycylglycine buffer would consist of significant amounts of both monomers and tetramers, thus allowing a sigmoidal response to the increase in protein concentration. The mass distribution of PFK in 0.1 M phosphate would contain a much greater proportion of tetramer, thus demonstrating a hyperbolic response to increased PFK concentrations. Not only do these results imply a relationship between enzyme activity and the aggregation state of PFK but also they reiterate that any studies on PFK, kinetic or physical, performed in the presence of phosphate will reflect the altered state of the self-association equilibrium. Analysis of the results of any such studies must consider these changes in the polymeric state of PFK before any conclusion can be derived and attributed to the intrinsic properties of the enzyme.

One cannot extrapolate the results of this analysis of ligand–PFK interactions to all ligands nor can this study preclude the possibility that the ligands studied could also have a kinetic effect on the enzyme. It remains possible for the ligands to exert an effect strictly through a kinetic mechanism. This mode of action could be observed via steady-state kinetics for the reaction would be kinetically controlled and the influence of quaternary structure would have no influence on the steady-state kinetic observations. However, in light of the results of the studies on ligand–PFK interactions, increasingly thorough studies are essential to separate the kinetics and allosteric effects of ligand binding to PFK.

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# pH and Temperature Effects on the Molecular Conformation of the Porcine Pancreatic Secretory Trypsin Inhibitor As Detected by Hydrogen-1 Nuclear Magnetic Resonance†

Antonio De Marco,* Enea Menegatti, and Mario Guarneri

ABSTRACT: $^1$H NMR spectra of the porcine pancreatic secretory trypsin inhibitor (PSTI) have been recorded vs. pH and temperature. Of the two tyrosines, one titrates with a p$K$ of 11.25, while the resonances from the other are pH insensitive in the investigated range $4.8 \leq pH \leq 12$. This is consistent with PSTI having one Tyr solvent exposed (Tyr-20) and the other buried (Tyr-31). The resonances from the lysyl $\epsilon$-$CH_2$ protons titrate with a p$K$ of 10.95. The titration is accompanied by a pronounced line broadening, which starts near pH 8.5. Between pH 11.5 and pH 12 the $\epsilon$-$CH_2$ resonances recover their low pH line width. Titration curves for the lysines and Tyr-20 reflect single proton ionization equilibria, suggesting that these residues do not interact among themselves. On the basis of double resonance experiments, combined with analysis of chemical shifts, spin–spin couplings, and line widths, all methyl resonances are identified and followed as functions of pH and temperature. The $\gamma$-$CH_3$ doublet from the N-terminal Thr-1 is assigned by comparison between spectra of forms I and II of the inhibitor, the latter lacking the first four residues of form I. The $\beta$-$CH_3$ resonance from Ala-7 is also assigned. Proton resonance parameters of methyl groups are shown to afford useful NMR probes for the characterization of local nonbonded interactions, microenvironments, and mobilities.

In recent years there has been an increasing interest in serine proteases and their inhibitors. Among their numerous functions, serine proteases appear to have an important role in tumor promotion and hormone action, activities which are blocked by protease inhibitors (Troll et al., 1970; Katz et al., 1977). Proteases also participate in the survival "SOS" mechanism in mutagenesis in *Escherichia coli* (Witkin, 1976), an action that is blocked by specific inhibitors as well (Meyn et al., 1977).

Protease inhibitors define a class of small proteins. They have become extremely useful for the study of structural dynamics of globular polypeptides. The Kunitz trypsin inhibitor from bovine pancreas (BPTI)[1] is so far one of the best char-

† From the Istituto di Chimica delle Macromolecole del CNR, 20133 Milano, Italy (A.D.M.), and the Istituto di Chimica Farmaceutica dell'Università di Ferrara, 44100 Ferrara, Italy (E.M. and M.G.). *Received June 2, 1981.* This work was supported in part by grants from Lepetit S.p.A., Milano, and from the Progetto Finalizzato del CNR Chimica Fine e Secondaria.

[1] Abbreviations: NMR, nuclear magnetic resonance; ppm, parts per million; TSP, sodium 3-(trimethylsilyl)(2,2,3,3-$^2H_4$)propionate; UV, ultraviolet spectroscopy; PSTI, pancreatic secretory trypsin inhibitor; BPTI, basic pancreatic trypsin inhibitor; HPI, pancreatic trypsin inhibitor from *Helix pomatia*; CTI, pancreatic trypsin inhibitor from cow colostrum; FID, free induction decay.

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