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ARTICLE *in* BIOCHEMISTRY · DECEMBER 1983

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Self-Association of Rabbit Muscle Phosphofructokinase: Role of Subunit Interaction in Regulation of Enzymatic Activity†

Michael A. Luther, Hiram F. Gilbert, and James C. Lee*

ABSTRACT: Phosphofructokinase (PFK) has been reversibly inactivated by oxidized glutathione. The physical properties of this inactivated form were characterized by sedimentation studies. In the presence of saturating amounts of fructose 6-phosphate and the nonhydrolyzable ATP analogue 5′-adenylyl imidodiphosphate, the inactivated PFK sediments as a 13.5S component. Sedimentation equilibrium study identifies it to be a tetramer with a molecular weight of 320 000. Sedimentation velocity studies in buffer alone at both pH 7.00 and pH 8.55 reveal that this inactivated enzyme can still undergo self-association with the same stoichiometry as that of the native enzyme, although the equilibrium constants are in favor of the formation of lower aggregates. The presence of allosteric activators, either ADP or cAMP, enhances the association of the inactivated PFK without changing its stoichiometry, an observation similar to that of the native enzyme. However, quantitatively the enhancement of association is not to the same extent as that of the native enzyme. Conversely, citrate favors the formation of smaller aggregates quantitatively similar to that of the native enzyme. Hence, this study shows that the oxidation state of the protein sulfhydryl groups influences the physical properties of PFK and the modulation of PFK activity is linked to subunit association–dissociation and quaternary structural changes.

Rabbit muscle phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) (PFK)[1] is an important glycolytic enzyme which is subjected to allosteric regulation. It has been demonstrated that a number of ligands can affect the enzymatic activity of PFK (Hanson et al., 1973; Hofer & Pette, 1968; Frieden et al., 1976; Goldhammer & Hammes, 1978). These ligands include substrates, products, H^+, divalent cations, AMP, cAMP, and other intermediary metabolites such as citrate and triose phosphates. The mechanism by which these ligands exert their effects has been the subject of intensive investigation.

Based mainly on kinetic observations the currently accepted model for the regulation of PFK is that it exists in two conformations, namely, an active R form and an inactive T form. It is assumed that the R and T forms represent two conformational isomers of the tetrameric enzyme (Goldhammer & Hammes, 1978; Roberts & Kellett, 1979, 1980a,b; Frieden et al., 1976). Frieden and co-workers further propose that the activation or inactivation of the enzyme depends on either the protonation or deprotonation of certain ionizable groups on this tetramer (Bock & Frieden, 1976a,b; Frieden et al., 1976; Pettigrew & Frieden, 1979a,b). Ligands are proposed to exert their influence by binding preferentially to either protonated (inactive) or unprotonated (active) forms of the enzyme, thus shifting the apparent pK of an important ionizable group. In this context, ATP is proposed to bind to the inactive T form, while F6P binds to the active R form. According to the proposed model, activators of PFK exert their effects by shifting the ratio of protonated to unprotonated forms of PFK; however, inhibitors such as citrate do not alter this ratio directly, but their effects are explained on the basis of induced differences in F6P binding. The physical state of these forms had not been identified until Hesterberg & Lee (1980) demonstrated that the active form sediments as a 12.4S particle. Subsequently, it was shown to be a tetramer (Hesterberg et al., 1981). An inactive tetramer has so far only been inferred as a result of computer simulation of sedimentation data (Hesterberg & Lee, 1981, 1982).

The effort to elucidate the molecular mechanism of regulation is further complicated by the fact that PFK is also capable of undergoing self-association (Parmeggiani et al., 1966; Ling et al., 1965; Leonard & Walker, 1972) in a rapid, dynamic equilibrium within the limits of resolution by sedimentation velocity (Hesterberg & Lee, 1981, 1982). This self-association equilibrium has been clearly demonstrated both qualitatively and quantitatively to be influenced by the presence of ligands (Parmeggiani et al., 1966; Lad et al., 1973; Aaronson & Frieden, 1972; Hill & Hammes, 1975; Reinhardt & Lardy, 1980; Hesterberg & Lee, 1982). The role of self-association in the regulation of PFK activity is still not defined, although in his model Frieden proposes that self-association plays no role in the mechanism of regulation due to the apparently slow rate constant governing the association (Bock & Frieden, 1976a,b; Frieden et al., 1976).

A major goal of this investigation is to provide physical evidence for the inactive form of PFK and to elucidate the role of self-association in the regulation of the enzymatic activity of PFK. A search was initiated to identify conditions that would lead to a reversible inhibition of PFK. Recently, Gilbert has shown that PFK can be reversibly inactivated by oxidized glutathione at low protein concentrations. This inhibition can be reversed upon the addition of DTT such that greater than 95% of the original activity returns (Gilbert, 1982). The physical state of this inactive form is not known. An effort was initiated to elucidate the physical properties and association behavior of this inactive form of PFK. These studies reveal the presence of an inactive tetramer that sediments as a 13.5S particle and is capable of undergoing association and

† From the E. A. Doisy Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri 63104 (M.A.L. and J.C.L.), and the Verna and Marrs McLean Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77030 (H.F.G.). *Received May 4, 1983.* This study was supported by National Institutes of Health Grants NS-14269 and AM-21489 (to J.C.L.) and GM-25347 and HL-28521 (to H.F.G.) and Grant Q-785 from the Robert A. Welch Foundation (to H.F.G.).

[1] Abbreviations: PFK, phosphofructokinase; GSSG, oxidized glutathione; TEMA buffer, 75 mM Tris–carbonate, 18 mM $MgCl_2$, 9 mM $(NH_4)_2SO_4$, and 3 mM EDTA; F6P, fructose 6-phosphate; AMP-PNP, 5′-adenylyl imidodiphosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

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dissociation as a function of protein concentration and substrates or effectors.

Materials and Methods

ATP, F6P, citrate, ADP, GSSG, and DTT were purchased from Sigma Chemical Co. cAMP, AMP-PNP, aldolase, glycerol-3-phosphate dehydrogenase–triosephosphate isomerase, and NADH were obtained from Boehringer-Mannheim. These were used without further purification.

PFK was purified, stored, and assayed as previously described (Hesterberg & Lee, 1981). The specific activity was ≥300 units/mg. The enzyme was equilibrated in the appropriate buffer by passage through a Sephadex G-25 column (0.9 × 5 cm), and the concentration of the enzyme was determined by the absorbance at 280 nm with an absorptivity coefficient of 1.07 L/(g·cm) (Hesterberg & Lee, 1981).

Inactivation of PFK was performed by the addition of oxidized glutathione at a final concentration of 10 mM to a solution of 1250 μg of PFK at 50 μg/mL equilibrated in 0.1 M Tris, 25 mM $MgSO_4$, and 1 mM EDTA at pH 8.55. At various time intervals, an aliquot was removed and assayed for PFK activity. The totally inactivated PFK was precipitated by 80% $(NH_4)_2SO_4$, and the excess GSSG was removed by passage through a Sephadex G-25 column (1.2 × 8.5 cm) equilibrated with 0.3 M Tris–PO_4 and 1 mM EDTA at pH 8.00 and then precipitated again by 80% $(NH_4)_2SO_4$ and stored at 5 °C. Control experiments showed no reactivation of PFK when the enzyme was stored under these conditions.

Reactivation of PFK was performed by addition of DTT at a final concentration of 100 mM to 1250 μg of inactivated PFK at 50 μg/mL equilibrated in 0.1 M Tris, 25 mM $MgSO_4$, and 1 mM EDTA at pH 8.55. At various time intervals an aliquot was removed and assayed for PFK activity. Reactivation was allowed to continue until 100% of the original activity had been recovered. The enzyme after reactivation was precipitated with 80% $(NH_4)_2SO_4$, and the excess DTT was removed by passage through a Sephadex G-25 column (1.2 × 8.5 cm) equilibrated with 0.3 M Tris–PO_4 and 1 mM EDTA at pH 8.00. The enzyme was then again precipitated with 80% $(NH_4)_2SO_4$ and stored at 5 °C.

Sedimentation velocity studies were carried out and analyzed by previously published procedures (Hesterberg & Lee, 1981). Weight-average sedimentation coefficients, $\bar{s}_{20,w}$, were determined from the centroid, a close approximation of the second moment, of the scanner-traced boundaries. The observed weight-average sedimentation coefficients were normalized to standard conditions by correcting for solvent density and viscosity. All experiments were performed in 75 mM Tris–carbonate, 18 mM $MgCl_2$, 9 mM $(NH_4)_2SO_4$, and 3 mM EDTA (TEMA buffer) at pH 7.00 or pH 8.55. The molecular weight of PFK in the presence of 1 mM F6P and 0.1 mM AMP-PNP was determined by the high-speed equilibrium method of Yphantis (1964) at 23 °C and 12000 rpm in TEMA buffer at pH 8.55. The concentration gradient was monitored by the photoelectric scanner at 280 nm. For all experiments, Kel-F coated aluminum double sector centerpieces with sapphire windows were used. The apparent partial specific volume of PFK in native condition is 0.730 (Hesterberg & Lee, 1982).

Results

Since the rabbit muscle PFK sample employed in this study is purified by a different procedure than that initially studied by Gilbert (1982), the inactivation of the enzyme by oxidized glutathione was monitored. The enzyme is inactivated by GSSG as shown in Figure 1. Approximately 90% of the

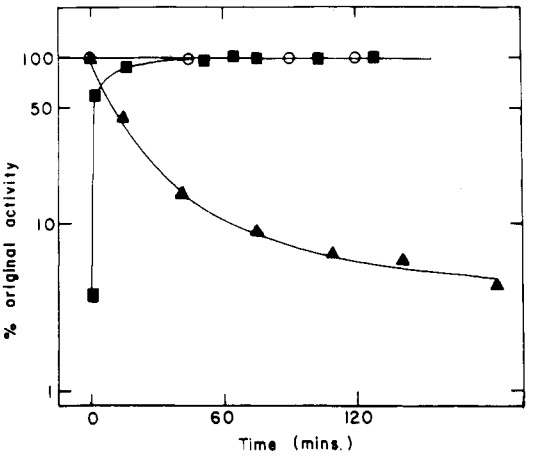

FIGURE 1: Inactivation and reactivation of PFK at pH 8.55 and 23 °C. PFK was inactivated by GSSG and reactivated by DTT. PFK concentration was 0.41 μg/mL in all assays, and 100% activity corresponds to a specific activity of 360 units/mg. The symbols and experimental conditions are (○) 0 mM GSSG, (▲) 10 mM GSSG, and (■) inactivated PFK in the presence of 100 mM DTT.

enzyme activity is lost in a rapid first-order reaction followed by a slower loss of residual activity at prolonged incubation times. Within 200 min of incubation essentially all of the original activity was inhibited. On the other hand, the control sample of enzyme treated in exactly the same manner showed no decrease in activity within the same period as shown in Figure 1. This latter observation on the control sample is identical with that reported previously (Hesterberg & Lee, 1980); however, it is significantly different from the report by Gilbert (1982) employing a commercially available PFK sample prepared by the procedure outlined by Ling et al. (1966). A control PFK sample prepared by this procedure and procured from Sigma showed a continuous decrease in activity in aerobic solutions as originally reported by Gilbert (1982). The cause of such quantitative differences between the two enzyme preparations is under further investigation.

The observed inactivation is totally reversible by the addition of DTT as shown in Figure 1. Essentially all of the original activity was recovered within 30 min. Nevertheless, it is essential to establish that the processes of inactivation and reactivation are completely reversible and that they have not altered the basic regulatory properties of PFK. Steady-state kinetics were monitored for both the native and reactivated samples of PFK. Effects of varying concentrations of F6P or ATP on the initial velocity were studied at both pH 8.55 and pH 7.00 in TEMA buffer. By the criterion of these simple kinetic measurements, the reactivated form of PFK behaves in an identical manner as the native form. Further analysis of the data indicated that the values for K_m are identical for both forms and at pH 8.55 they assume values of approximately 70 μM and 75 μM for ATP and F6P, respectively, as summarized in Table I. These values are in good agreement with the literature values (Kee & Griffin, 1972; Hanson et al., 1973; Hesterberg & Lee, 1980). It may be concluded that the processes of inactivation and reactivation have not generated detectable irreversible alterations in the affinity of PFK for its substrates or in its catalytic ability. Additional kinetic measurements were conducted to test the effects of an activator, cAMP, and an inhibitor, citrate, on the activity of the native and reactivated forms of PFK. As summarized in Table I, these effectors modified the activity of both forms of PFK to the same extent, hence, suggesting that the regulatory properties of PFK have not been irreversibly altered by the processes of chemical modification.

Table I: Steady-State Kinetic Parameters for Native and Reactivated PFK[a]

condition	K_m (μM) ATP	K_m (μM) F6P	V_{max} (units/mg)[b]
(A) Native PFK			
at pH 7.0			
no effector	90 ± 5	93 ± 5	360
+300 μM cAMP	75	82	360
+10 mM citrate	97	160	360
at pH 8.55			
no effector	71	79	370
+300 μM cAMP	69	62	360
+10 mM citrate	78	138	340
(B) Reactivated PFK			
at pH 7.00			
no effector	97	92	360
+300 μM cAMP	72	87	370
+10 mM citrate	105	150	360
at pH 8.55			
no effector	73	75	370
+300 μM cAMP	71	68	380
+10 mM citrate	77	124	340

[a] All experiments were performed in TEMA buffer at 23 °C. [b] A unit is defined as 1 μmol of substrate converted to product per min.

Having established the reversibility of the inactivation process, it is of interest to identify the physical state of the inactive form, so as to further elucidate the molecular mechanism(s) of modulation of 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 8.55 and pH 7.00, 23 °C, in TEMA buffer within the protein concentration range of 10–500 μg/mL. Under all experimental conditions and for all PFK forms employed the systems were tested for rapid, dynamic equilibrium by the three criteria adopted previously, namely, measurement of $s_{20,w}$ as a function of ω^2, independence of $s_{20,w}$ as a function of time of dilution from a stock solution of higher protein concentration, and the adherence of the sedimentation patterns as predicted by Gilbert (1963) for a self-associating system in a rapid, dynamic equilibrium. On the basis of these criteria it was established that the data are amenable to such an analysis. When the inactivated form of PFK was subjected to sedimentation analysis, it was observed that the values of $\bar{s}_{20,w}$ were lower than that of the native enzyme at pH 7.00 and pH 8.55 as shown in Figure 2. The deviation increased with increasing protein concentration, indicating either that the inactivated form of PFK has a different mode of association or that the tendency for self-association has decreased. Upon reactivation, however, PFK exhibited a sedimentation behavior identical with that of the native form at both pH 7.00 and pH 8.55 as shown in Figure 2, implying again that the process of inactivation is completely reversible.

The results from sedimentation experiments were further analyzed by theoretical calculations and curve fitting by using previously published procedures (Hesterberg & Lee, 1981). Results from such an analysis are summarized in Table II. The simplest model that fits the data the best for both the native and the reactivated forms of PFK at either pH yields a stoichiometry of $M_1 \rightleftharpoons M_4 \rightleftharpoons M_{16}$ where M_4 is the tetramer assuming a sedimentation coefficient, s_4^0, of 13.5 S. The equilibrium constants calculated are in very good agreement with published results (Hesterberg & Lee, 1982). However, the simplest model for the inactivated form of PFK at either pH is $M_1 \rightleftharpoons M_2 \rightleftharpoons M_4 \rightleftharpoons M_{16}$ with s_4^0 assuming a value of 13.5 S. The major effect of inactivation by GSSG is on the apparent equilibrium constants K_2^{app} and K_{16}^{app}. The value of K_4^{app}, however, is not significantly altered. These sedimentation results show that while both forms are capable of undergoing self-association, the inactivated form exhibits a lower tendency to aggregate to the 16-mer with a concomitant increase in its tendency to form dimers. The overall hydrodynamic properties of the aggregates of both forms are the same since the values needed to fit both sets of data are identical.

The self-association of PFK is strongly influenced by the presence of ligands (Parmeggiani et al., 1966; Lad et al., 1973;

Table II: Summary of Self-Association Studies on Native, Inactive and Reactivated PFK[a]

ligands	s_4^0	stoichiometry	K_2^{app} (mL/mg)	K_4^{app} $(mL/mg)^3$	K_{16}^{app} $(mL/mg)^{15}$	σ
(A) Native and Reactivated PFK						
pH 7.00						
no effector	13.5	1-4-16		5.06×10^5	3.25×10^{23}	0.31
10 mM citrate	13.5	1-2-4-16	457.45	4.93×10^5	4.63×10^{20}	0.16
0.1 mM ADP	13.5	1-4-16		6.20×10^8	3.99×10^{35}	0.33
0.1 mM cAMP	13.5	1-4-16		1.41×10^8	9.95×10^{32}	0.21
0.1 mM AMP-PNP, 1 mM F6P	12.4					
pH 8.55						
no effector	13.5	1-4-16		1.15×10^7	4.60×10^{28}	0.29
10 mM citrate	13.5	1-2-4-16	290.10	4.25×10^5	1.65×10^{22}	0.21
0.1 mM ADP	13.5	1-4-16		6.20×10^8	4.00×10^{35}	0.33
0.1 mM AMP-PNP, 1 mM F6P	12.4					
(B) Inactivated PFK						
pH 7.00						
no effector	13.5	1-2-4-16	82.50	8×10^5	6.59×10^{22}	0.25
10 mM citrate	13.5	1-2-4-16	457.45	4.93×10^5	4.63×10^{20}	0.16
0.1 mM ADP	13.5	1-4-16		7.05×10^6	5.05×10^{23}	0.36
0.1 mM cAMP	13.5	1-4-16		1.37×10^6	3.11×10^{23}	0.11
0.1 mM AMP-PNP, 1 mM F6P	13.5					
pH 8.55						
no effector	13.5	1-2-4-16	105.63	3.92×10^6	9.79×10^{25}	0.24
10 mM citrate	13.5	1-2-4-16	344.14	4.94×10^5	1.36×10^{19}	0.12
0.1 mM ADP	13.5	1-4-16		7.05×10^6	5.05×10^{23}	0.36
0.1 mM AMP-PNP, 1 mM F6P	13.5					

[a] All experiments were conducted in TEMA buffer at 23 °C.

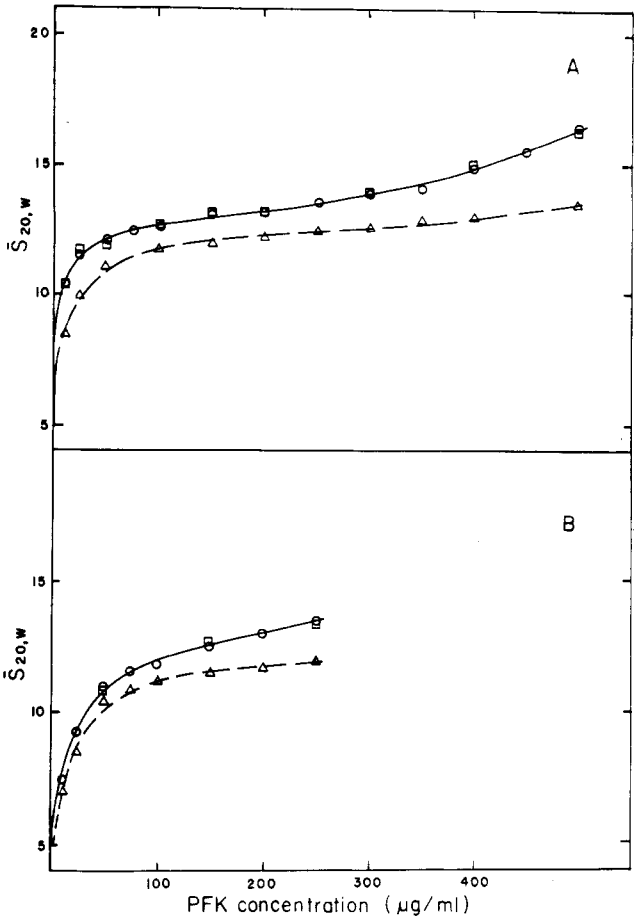

FIGURE 2: Effect of pH on the $\bar{s}_{20,w}$ as a function of PFK concentration in TEMA buffer at 23 °C. (A) pH 7.00; (B) pH 8.55. The symbols and state of the enzyme are (○) native, (△) inactivated, and (□) reactivated. The lines represent the theoretical fit of the experimental data. The symbols and corresponding conditions are the following: (—) using the association model of $M_1 \rightleftharpoons M_4 \rightleftharpoons M_{16}$ and s_4^0 of 13.5 S; (---) using the association model of $M_1 \rightleftharpoons M_2 \rightleftharpoons M_4 \rightleftharpoons M_{16}$ and s_4^0 of 13.5 S. The data points are the average of multiple data sets.

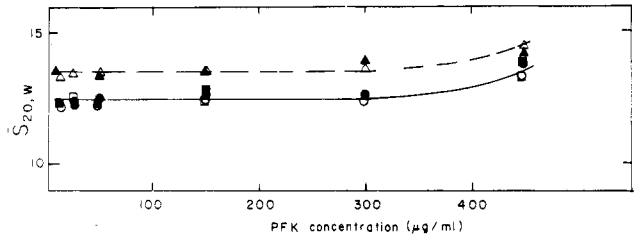

FIGURE 3: Effect of 0.1 mM AMP-PNP and 1.0 mM F6P on the $\bar{s}_{20,w}$ as a function of PFK concentration in TEMA buffer, 23 °C. Symbols are the same as described in Figure 2. Open and closed symbols are data obtained at pH 7.00 and pH 8.55, respectively.

Aaronson & Frieden, 1972; Hill & Hammes, 1975; Reinhart & Lardy, 1980; Hesterberg & Lee, 1982). It is, therefore, important to characterize the effects of ligands on the sedimentation behavior of the inactivated and reactivated forms of PFK. In the presence of 1 mM F6P and 0.1 mM AMP-PNP at either pH 7.00 or pH 8.55 and within the concentration range of 12.5–150 μg/mL, native PFK sediments essentially as a single component with a sedimentation coefficient of 12.4 S, as shown in Figure 3. With increasing protein concentration there is an indication of the presence of larger aggregates as demonstrated by a continuous increase in the value of $\bar{s}_{20,w}$. Inactivated PFK exhibits a significantly different sedimentation behavior, as shown in Figure 3. Within the protein concentration range of 12.5–150 μg/mL, GSSG-inactivated PFK sediments with a sedimentation coefficient of 13.5 S with no detectable amounts of smaller or larger aggregates. At higher protein concentrations the observed values of $\bar{s}_{20,w}$ increase with increasing concentrations. The presence of a 13.5S component as shown in this study is the first direct physical evidence for this inactive form of PFK, whereas in previous publications the presence of this component has only been implied as a consequence of computer curve fitting of sedimentation data (Hesterberg & Lee, 1981, 1982). Reactivated PFK demonstrates exactly the same sedimentation behavior as native PFK, as shown in Figure 3, again demonstrating the complete reversibility of the inactivation process. When the results of the inactivated form in the absence and presence of substrates are compared, it is evident that the presence of substrates enhances the formation of the larger aggregates of PFK, an observation qualitatively similar to that of the native or reactivated forms.

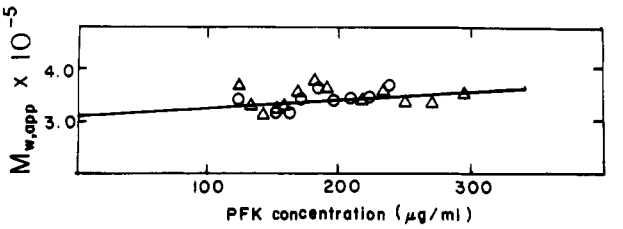

FIGURE 4: Concentration dependence of the weight-average molecular weight in the presence of 1.0 mM fructose 6-phosphate and 0.1 mM AMP-PNP. Loading concentrations of inactivated PFK were (○) 100 and (△) 150 μg/mL.

To determine if this 13.5S component does truly represent an inactive tetrameric form of PFK, sedimentation equilibrium experiments in the presence of 0.1 mM AMP-PNP and 1.0 mM F6P were conducted to monitor the molecular weight of PFK at loading concentrations of 100 and 150 μg/mL. There is a slight concentration dependence in apparent weight-average molecular weight with an extrapolated value of 320 000 at infinite dilution, as shown in Figure 4. The overlapping of the apparent molecular weights regardless of initial loading concentrations indicates that the system is in equilibrium and that the small increase in molecular weight with increasing PFK concentration is due to a further aggregation of PFK tetramers, a conclusion that is consistent with the sedimentation velocity data in Figure 3. These sedimentation studies provide evidence that the 13.5S inactive form is a conformational isomer of the tetrameric species of PFK.

The effect of ADP, an allosteric activator, on the self-association of the three forms of PFK was determined. As shown in Figure 5A in the presence of 0.10 mM ADP, at pH 7.00, 23 °C, the self-association of native PFK is greatly enhanced over that in buffer alone. The model that best fits the data for the native enzyme remains the same with negligible amount of PFK dimer, and s_4^0 assumes a value of 13.5 S. This is in good agreement with results previously reported (Hesterberg & Lee, 1982). The reactivated form behaves identically with that of native PFK, as shown by Figure 5A; however, the inactivated form behaves differently. While ADP does enhance the self-association of this form, it is not to the same degree as that for the native or reactivated forms of PFK, implying that the inactivated form of PFK has a lower propensity to self-associate. When these same experiments were repeated at pH 8.55, ADP did not exert any further enhancement of the association of all forms of PFK, when compared to the results at pH 7.00 in the presence of ADP as shown in Figure 5A. These results imply that the effect of ADP observable at pH 7.00 is at its maximum. The effect of another allosteric activator, cAMP, on the self-association

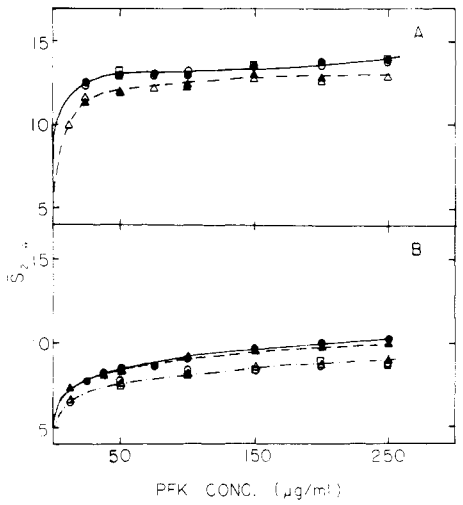

FIGURE 5: Effect of metabolites on the $\bar{s}_{20,w}$ as a function of PFK concentration in TEMA buffer at 23 °C. All lines represent the theoretical fit of the experimental data. The symbols and state of the enzyme are (○) native, (△) inactivated, and (□) reactivated. Open and closed symbols are data obtained at pH 7.00 and pH 8.55, respectively. The symbols and experimental conditions are the following: (A) 0.1 mM ADP, (—) native and reactivated PFK and (---) inactivated PFK. The association model used in theoretical fitting is $M_1 \rightleftharpoons M_4 \rightleftharpoons M_{16}$ and s_4^0 of 13.5 S. (B) 10 mM citrate, (—) native and reactivated PFK at pH 8.55, (---) inactivated PFK at pH 8.55, and (···) all forms of PFK at pH 7.00. The association model used in theoretical fitting is $M_1 \rightleftharpoons M_2 \rightleftharpoons M_4 \rightleftharpoons M_{16}$ and s_4^0 of 13.5 S.

of the various forms was also monitored. Quantitatively, PFK exhibits very similar behavior in 0.10 mM cAMP at pH 7.00, 23 °C, as in the presence of ADP. Values for s_4^0 and apparent equilibrium constants are summarized in Table II. The exact equivalence of apparent equilibrium constants obtained for the measurements is the result of the use of a finite stepsize in the data-fitting algorithm.

The effect of the allosteric inhibitor, citrate, on the self-association of various forms of PFK was determined, also. Results on native PFK in the presence of 10 mM citrate at pH 7.00 and pH 8.55 in TEMA buffer indicated a decrease in the values of $\bar{s}_{20,w}$, as shown in Figure 5B. The data can best be fitted to a model of $M_1 \rightleftharpoons M_2 \rightleftharpoons M_4 \rightleftharpoons M_{16}$, in good agreement with a published report (Hesterberg & Lee, 1982). s_4^0 assumes a value of 13.5 S. The behavior of reactivated PFK is indistinguishable from that of the native enzyme, as shown in Figure 5B. At either pH 7.00 or pH 8.55, the inactivated form behaves identically with that of the native and reactivated PFK, although at higher protein concentrations at pH 8.55 the inactivated PFK shows a slightly decreased propensity to aggregate. It is evident that the effect of citrate on dissociation at pH 8.55 is not as pronounced as that at pH 7.00. All the apparent equilibrium constants from the theoretical curve fitting are summarized in Table II.

Discussion

On the basis of the results of kinetic measurements there is a general consensus in the literature that the regulatory properties of PFK can be fitted to a Monod–Wyman–Changeux model in which an active species, R, and an inactive species, T, are in equilibrium (Goldhammer & Hammes, 1978; Roberts & Kellett, 1979, 1980a,b; Bock & Frieden, 1976a,b; Frieden et al., 1976). The details of the model may vary depending on the laboratory, and the direct correspondence of species remains to be demonstrated. However, one common feature is that all of these models only consider regulation within the tetrameric form of the enzyme, although it is known to undergo association–dissociation. The studies reported in the literature were generally conducted under conditions in which PFK exists predominantly as a tetramer at the start of the experiment, and furthermore, it was assumed that there was no change in the aggregation state of PFK during subsequent perturbation of the system, e.g., addition of ligands or dilution of enzyme solution. Although the results of these data analyses are internally consistent, direct evidence for the physical nature of these R and T states is not available. The major thrusts of this report are 2-fold, namely, to provide physical evidence for an active and an inactive tetrameric PFK and to quantitatively probe the effects of regulatory metabolites on the aggregation of this inactive form of PFK.

Gilbert (1982) reported that muscle PFK can be reversibly inactivated by oxidized glutathione. The physical properties of this inactivated form have now been characterized. In combination with knowledge acquired from previous studies from this laboratory it may be concluded that the two PFK states representing an active and an inactive state have now been identified. In the presence of saturating amounts of F6P and AMP-PNP, the active form of the native enzyme is a tetramer which sediments as a 12.4S component (Hesterberg & Lee, 1980; Hesterberg et al., 1981). However, in this study under identical conditions an inactive form has been demonstrated to be a tetramer which sediments as a 13.5S particle. Hence, it is proposed that the 12.4S tetrameric PFK corresponds to the active R state and the 13.5S tetramer to one of the inactive species in the T state. Let us examine the evidence that led to this conclusion and the validity of proposing the 13.5S tetramer as an inactive form of PFK.

The tetrameric nature of the two proposed forms of PFK was established by sedimentation equilibrium experiments in this study and previously published results (Hesterberg et al., 1981). Sedimentation velocity studies, however, identify differences in the hydrodynamic properties of the active and inactive forms. The active form probably assumes a more relaxed structure with a sedimentation coefficient of 12.4 S, whereas the inactive form is a more compact one with a sedimentation coefficient of 13.5 S. An important observation from this study is that this inactivated form can be totally reactivated. The inactivation reaction is completely reversible, and the enzyme shows no evidence of irreversible modifications as indicated by the results obtained by steady-state kinetic measurements in the presence and absence of effectors (Table I) and from sedimentation velocity experiments on the native and reactivated PFK (Figure 3). Thus, the results of these studies provide pieces of evidence that fulfill one of the necessary, but minimum, criterion in assessing the biological relevance of this report, i.e., the complete reversibility of the reaction. Having satisfied this criterion, it then leads to the question of the validity in assigning these forms to active and inactive states of PFK. The evidence available to support the assignment of a 12.4S tetramer as the active form is numerous. These include results from electron microscopy, active enzyme centrifugation, sedimentation velocity, and equilibrium (Hesterberg & Lee, 1979, 1980; Hesterberg et al., 1981). The presence of a 13.5S tetrameric PFK has so far only been inferred as a consequence of computer fitting of sedimentation velocity experiments (Hesterberg & Lee, 1981, 1982). A value of 13.5 S is required to best fit the data in the presence of activators, inhibitors, or buffer alone. However, in the presence of either F6P or ATP, or a combination of both F6P and AMP-PNP, a value of 12.4 S is essential for obtaining the best fit of the data, thus demonstrating a high degree of specificity in the ability of substrates or substrate analogues to induce

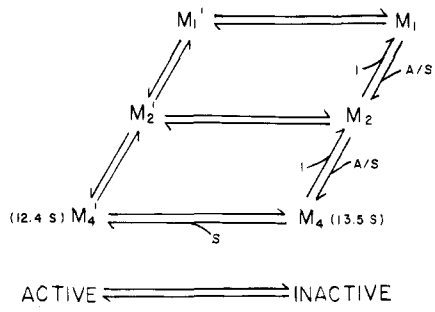

FIGURE 6: Proposed model for the regulation of PFK. M_x and M_x' represent species in the inactive and active conformation, respectively. The subscript indicates the number of subunits in each species. A, I, and S represent activators, inhibitors, and substrates, respectively.

such structural changes. Hence, these observations are consistent with the proposed Monod–Wyman–Changeux model that substrates would shift the equilibrium in favor of the R state. Furthermore, the verification of the presence of these two forms of tetramer serves as strong support for the analytical effectiveness of the computer fitting procedure employed.

This study provides physical evidence for the existence of an inactive tetrameric PFK with a sedimentation coefficient of 13.5 S. At present, one can at least conclude that the hydrodynamic properties of this chemically inactivated form of PFK are similar to those inferred in the native unmodified protein. Definitive characterization is still not available to establish a direct correspondence of the chemically modified and unmodified 13.5S tetramer. All the metabolites tested in this study interact with the oxidized, inactive PFK and induce the enzyme to associate or dissociate in a manner qualitatively very similar to that observed in the native enzyme. These facts provide additional evidence that the assignment of the 13.5S tetramer as one of the species in the T state is reasonable.

Having established that the 13.5S component is an inactive tetrameric PFK, it is of interest to examine the propensity of this component to undergo association–dissociation to provide evidence for the potential role of subunit association in the regulation of PFK activity. Previously published results from this laboratory have established that PFK at low protein concentrations undergoes a rapid dynamic association–dissociation with a stoichiometry of $M_1 \rightleftharpoons M_2 \rightleftharpoons M_4 \rightleftharpoons M_{16}$ (Hesterberg & Lee, 1981). It is proposed that the 12.4S tetramer is probably the only active form at low protein concentrations, whereas the monomeric and dimeric species are inactive. This is based on the fact that within an experimental uncertainty of ±10%, the enzymatic activity observed in active enzyme centrifugation studies accounts for all of that in steady-state kinetic experiments. If the system indeed is regulated at least partially by subunit association–dissociation, then it can be expected that kinetic inhibitors will favor dissociation of PFK, whereas activators and substrates should enhance the formation of higher aggregates. Such predictions are indeed substantiated by reports in the literature (Lad et al., 1973) and have been quantitatively characterized by this laboratory (Hesterberg & Lee, 1982). However, these studies raise a question about the state of the monomer and dimer: Do they exist in the active or inactive form? Results from this study provide evidence that the inactivated form can also undergo rapid dynamic association–dissociation. The overall stoichiometry remains the same as the native PFK, namely, $M_1 \rightleftharpoons M_2 \rightleftharpoons M_4 \rightleftharpoons M_{16}$, although the equilibrium constants governing these steps are lower. The effects of ligands on the association–dissociation of the inactivated PFK were monitored in this study. Similar to the observed effects on native PFK, the stoichiometry of the reaction remains the same, and specific ligands perturb the various equilibrium constants to different extents. However, in all cases tested the equilibrium constant governing each reversible step is lower than the corresponding equilibrium constant for native PFK. In the presence of activators, e.g., ADP, cAMP, and low concentrations of H^+, the association of subunits to higher aggregates is favored. Conversely, the presence of the allosteric inhibitor citrate and high concentrations of H^+ favor the formation of smaller aggregates. In all cases tested, the value of s_4^0 that best fits the data is 13.5 S, an observation similar to that of native PFK under identical conditions. However, the presence of saturating concentrations of substrates does not induce the formation of a 12.4S tetramer as observed for the native enzyme. Nevertheless, substrates do enhance the association of inactivated PFK subunits, suggesting that they still bind to the inactivated enzyme. It is evident that just like the native enzyme the inactivated PFK is capable of undergoing association–dissociation, but unlike the native enzyme it cannot undergo the conversion from an inactive 13.5S tetramer to the active 12.4S one. Thus, it is proposed that rabbit muscle PFK undergoes structural changes as shown in Figure 6. This study and other published results from this laboratory have identified the active species of M_4' and the inactive species of M_4, M_2, and M_1. There is as yet no direct evidence for the presence of monomeric and dimeric PFK in the active conformation. However, the observed decreased propensity for the inactivated form of PFK to associate implies either that the oxidation of sulfhydryl groups in PFK is the cause or that the inactive form of PFK does indeed exhibit a weaker association. If the latter interpretation is correct, then the apparent stronger association demonstrated by native PFK may imply that there is a parallel association–dissociation interaction among PFK subunits in its active conformation. There are currently no experimental results to differentiate these possibilities.

The significance of this study is the establishment of equilibria among the inactive species and that of the active form. Activators and inhibitors perturb the equilibria among the inactive species, whereas only substrates can favor the formation of the active form. This is the first physical evidence to link association–dissociation of PFK subunits to conformational changes between active and inactive tetrameric PFK. Since these events are linked by reversible equilibrium processes, perturbation on any part of this network of equilibria will ultimately be reflected in the amount of active tetrameric PFK present, and thus the enzymatic activity. The quantitative significance of association–dissociation and conversion between the active and inactive tetrameric PFK in the regulation of enzymatic activity remains to be defined; however, from the thermodynamic viewpoint the associate–dissociation of PFK subunits must play a role in the regulation of PFK.

Registry No. PFK, 9001-80-3; GSSG, 27025-41-8; F6P, 643-13-0; AMP-PNP, 25612-73-1; ATP, 56-65-5; ADP, 58-64-0; cAMP, 60-92-4; citrate, 77-92-9.

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Physical Association of a DNA Polymerase Stimulating Activity with a Ribonuclease H Purified from Yeast†

Robert Karwan, Hans Blutsch, and Ulrike Wintersberger*

ABSTRACT: From the yeast *Saccharomyces cerevisiae* we have purified by three consecutive column-chromatographic steps a ribonuclease H activity to apparent homogeneity. The enzyme, a single polypeptide chain of molecular weight around 68 000, is optimally active at neutral pH and at a magnesium ion concentration of 10 mM and is sensitive to *N*-ethylmaleimide. It degrades the RNA strand of a DNA-RNA hybrid in an endonucleolytic mode and hydrolyzes neither double- or single-stranded DNA nor single-stranded RNA. The enzyme is capable of stimulating in vitro DNA synthesis by yeast DNA polymerase A up to more than 50-fold. This effect is strongly dependent on the relative amounts of primer template, DNA polymerase, and ribonuclease H in the assay mixtures. Yeast DNA polymerase B and *Escherichia coli* DNA polymerase I are barely stimulated under our assay conditions.

Whereas for bacteria and their phages combined biochemical and genetic efforts have led to a fairly clear picture of the process of DNA replication, our knowledge of this fundamental mechanism in eukaryotes is less developed (Kornberg, 1980, 1982). In vitro studies are most advanced for viral DNA synthesis in animal cells [see, e.g., Winnacker (1978) and Waldeck et al. (1979)] although the proteins that are involved are only partly characterized. Because of the accessibility of yeast to simple genetic methods, we have earlier chosen this eukaryotic microorganism for biochemical studies of proteins involved in DNA synthesis. Three DNA polymerases had been characterized (Wintersberger, 1978), and DNA synthesis had been studied in isolated nuclei (Wintersberger, 1976). In order to find further proteins participating in DNA replication, repair, or recombination of yeast, we have undertaken a search for DNA-binding proteins able to stimulate in vitro DNA synthesis by yeast DNA polymerase A. Several such proteins from other organisms had been described (Hubermann et al., 1971; Banks & Spanos, 1975; Herrick et al., 1976; Otto et al., 1977; Duguet et al., 1977; Blue & Weissbach, 1978; Ganz & Pearlman, 1980; Riva et al., 1980). From yeast, a factor with a molecular weight of 37 000 that stimulated DNA synthesis on single-stranded DNA in the absence of a primer was isolated by Chang and co-workers (Chang et al., 1978). We describe here another DNA-binding protein from *Saccharomyces cerevisiae* that stimulates in vitro DNA synthesis on gapped double-stranded templates by DNA polymerase A.

During our attempts to isolate this protein, we noticed the copurification of a ribonuclease activity, which specifically degraded RNA in DNA-RNA hybrid structures, a ribonuclease H (RNase-H).[1] Thus, the question arose whether

† From the Institute for Tumor Biology/Cancer Research (R.K. and U.W.) and the Institute of Molecular Biology (H.B.), University of Vienna, A-1090 Vienna, Austria. *Received February 28, 1983.* This work was supported by Grant 4326 to U.W. from the Fonds zur Förderung der Wissenschaftlichen Forschung in Österreich.

[1] Abbreviations: DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid disodium salt; GF/C, glass microfiber filters; NEM, *N*-ethylmaleimide; PEI, poly(ethylenimine); SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; RNase-H, ribonuclease H; DNase I, deoxyribonuclease I.

0006-2960/83/0422-5500$01.50/0