Specific, Reversible Inactivation of Phosphofructokinase by Fructose-1,6-bisphosphatase. Involvement of Adenosine 5′-Triphosphate, Oleate, and 3-Phosphoglycerate†

Richard T. Proffitt, L. Sankaran, and Burton M. Pogell*

ABSTRACT: Optimal conditions necessary for the reversible inactivation of crystalline rabbit muscle phosphofructokinase by homogeneous rabbit liver fructose-1,6-bisphosphatase have been studied. At higher enzyme levels (to 530 μg/ml of phosphofructokinase) the two proteins were mixed and incubated in a pH 7.5 buffer composed of 50 mM Tris-HCl, 2 mM potassium phosphate, and 0.2 mM dithiothreitol. Aliquots were removed at various times and assayed for enzyme activity. A time dependent inactivation of phosphofructokinase caused by 1–2.3 times its weight of fructose-1,6-bisphosphatase was observed at 30, 23, and 0 °C. This inactivation did not require the presence of adenosine 5′-triphosphate or Mg^{2+} in the incubation mixture, but an adenosine 5′-triphosphate concentration of 2.7 mM or greater was required in the assay to keep phosphofructokinase in an inactive form. A mixture of activators (inorganic phosphate, $(NH_4)_2SO_4$, and adenosine 5′-monophosphate), when added to the assay cuvette, restored nearly all of the expected enzyme activity. Incubations with other proteins, including aldolase, at concentrations equal to or greater than the effective quantity of fructose-1,6-bisphosphatase had no inhibitory effect on phosphofructokinase activity. Removal of tightly bound fructose 1,6-bisphosphate from phosphofructokinase could not explain this inactivation, since several analyses of crystalline phosphofructokinase averaged less than 0.1 mol of fructose 1,6-bisphosphate/320 000 g of enzyme. Furthermore, the inactivation occurred in the absence of Mg^{2+}, where the complete lack of fructose-1,6-bisphosphatase activity was confirmed directly. At lower phosphofructokinase concentrations (0.2–2 μg/ml) the inactivation was studied directly in the assay cuvette. Higher ratios of fructose-1,6-bisphosphatase to phosphofructokinase were necessary in these cases, but oleate and 3-phosphoglycerate acted synergistically with lower amounts of fructose-1,6-bisphosphatase to cause inactivation. The inactivation did not occur when high concentrations of fructose 6-phosphate were present in the assay, or when the level of adenosine 5′-triphosphate was decreased. However, the inactivation was found at pH 8, where the effects of allosteric regulators on phosphofructokinase are greatly reduced. Experiments with rat liver phosphofructokinase showed that this enzyme was also subject to inhibition by rabbit liver fructose-1,6-bisphosphatase under conditions similar to those used in the muscle enzyme studies. Attempts to demonstrate direct interaction between phosphofructokinase and fructose-1,6-bisphosphatase by physical methods were unsuccessful. Nevertheless, our results suggest that, under conditions which approximate the physiological state, the presence of fructose-1,6-bisphosphatase can cause phosphofructokinase to assume an inactive conformation. This interaction may have a significant role in vivo in controlling the interrelationship between glycolysis and gluconeogenesis.

Phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) catalyzes the formation of Fru-1,6-P_2 from Fru-6-P and ATP and is considered to be a major control point for regulation of glycolysis (Bloxham and Lardy, 1973; Ramaiah, 1974). On the other hand, fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) catalyzes the hydrolysis of Fru-1,6-P_2 to Fru-6-P and is a major regulatory enzyme for gluconeogenesis (Pontremoli and Horecker, 1971; Pogell, 1975). Both enzymes are subject to control by a number of effectors including AMP, ATP, oleate, 3-P-glycerate, and Fru-1,6-P_2 (Kemp, 1971; Passonneau and Lowry, 1962; Taketa and Pogell, 1965; Pogell et al., 1971; Carlson et al., 1973). Together these two enzymes form a "futile cycle" which, if uncontrolled, results in a wasteful loss of ATP (Scrutton and Utter, 1968). Indeed, substrate cycling has been shown to exist in rat liver (Clark, et al., 1974) and may be a possible mechanism for heat generation in bumblebee flight muscle (Clark et al., 1973).

Several recent reports have implied that a direct interaction between PFK[1] and FDPase is involved in regulating the "futile cycle" which these enzymes form. El-Badry et al. (1973) have shown that sheep heart PFK was inhibited directly in assays by FDPase or aldolase. They felt that these observations could be explained by the removal of Fru-1,6-P_2 bound to PFK with the resultant conversion of PFK to an inactive form. In more recent studies, Uyeda and Luby (1974) reported that FDPase from chicken liver and rabbit muscle inhibited PFK from both sources, as well as enhancing inhibition by ATP or 3-P-glycerate. They concluded that there was evidence for a direct protein-protein interaction between the two enzymes from studies including specific quenching by FDPase of the fluorescence of PFK-ANS complexes, the absence of inhibition by FDPase derivatives with higher catalytic activity, and the

† From the Department of Microbiology, St. Louis University School of Medicine, St. Louis, Missouri 63104. *Received December 22, 1975.* This investigation was supported by Grant CA-12080 and Postdoctoral Fellowship CA-05331 (to R.T.P.), awarded by the National Cancer Institute, Department of Health, Education, and Welfare.

[1] The abbreviations used are: PFK, phosphofructokinase; FDPase, fructose-1,6-bisphosphatase; ANS, 8-anilino-1-naphthalenesulfonate; Fru-6-P, fructose 6-phosphate; Fru-1,6-P_2, fructose 1,6-bisphosphate; ATP, adenosine 5′-triphosphate; AMP, adenosine 5′-monophosphate; 3-P-glycerate, 3-phosphoglycerate; NADH, reduced nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Glc-6-P, glucose 6-phosphate; P_i, inorganic phosphate; CoA, coenzyme A; DTT, dithiothreitol.

protection of chicken liver PFK against cold inactivation by FDPase (Kono and Uyeda, 1974). Along a similar line, Pogell et al. (1968) showed that rabbit muscle PFK could prevent and reverse the AMP inhibition of rabbit liver FDPase. More recently, PFK has been shown to act as a protein carrier for oleate and to enhance activation of FDPase by this fatty acid (Carlson et al., 1975).

In this communication we report the reversible inactivation of PFK by FDPase under conditions that approach the physiological state. Our results are consistent with the hypothesis that FDPase can inactivate PFK under the appropriate conditions by causing it to assume an inactive conformation. A preliminary report of this work has been presented (Sankaran et al., 1975a).

Materials and Methods

Materials. Sodium salts of Fru-6-P (grade I), Fru-1,6-P_2, ATP, AMP, NADH, $NADP^+$, 3-P-glycerate, P-enolpyruvate, oleate, and EDTA, the ammonium salt of 8-anilino-1-naphthalenesulfonate, and lactic dehydrogenase (rabbit muscle, type II, 715 units/mg), pyruvate kinase (rabbit muscle, type II, 645 units/mg), glucose-6-phosphate isomerase (yeast, grade III, 630 units/mg), glucose-6-phosphate dehydrogenase (bakers yeast, type VII, 390 units/mg), aldolase (rabbit muscle, 18 units/mg), and bovine serum albumin were obtained from Sigma Chemical Co. The sources for other materials were Worthington, DNase II; Calbiochem, dithiothreitol; Pharmacia, CNBr-activated Sepharose 4B; and Bio-Rad Laboratories, Affi-Gel 10. All other chemicals were of analytical quality. Distilled deionized water was used to prepare all solutions.

Fructose-1,6-bisphosphatase. The preparation of homogeneous rabbit liver FDPase having high neutral activity has been described (Carlson et al., 1973; Ulm et al., 1975). The FDPase used in this work showed a specific activity of 25 when assayed at pH 7.3 and 30 °C according to the published procedure (Carlson et al., 1973). When necessary, concentrated enzyme in 5 mM sodium malonate (pH 6.0) was diluted with 50 mM Tris-HCl (pH 7.2) containing 0.5 M KCl. The preparation of "alkaline" FDPase has also been described (Sarngadharan et al., 1970).

Phosphofructokinase. Crystalline rabbit muscle PFK (type III) was obtained as an ammonium sulfate suspension from Sigma Chemical Co. Ammonium sulfate was routinely removed by dissolving the crystalline enzyme in 2–3 volumes of 10 mM potassium phosphate (pH 8)–0.1 mM DTT buffer and dialyzing overnight against 5000–6000 volumes of the same buffer. The protein concentration of the dialyzed material was 3.5–6.0 mg/ml by $A_{280/260}$ spectrophotometric determination (Layne, 1957), and the initial specific activity was approximately 100 when assayed at pH 8.0 and 30 °C. PFK was used within 5 days after dialysis. When necessary, dialyzed PFK was further diluted with dialysis buffer just before use. Purified rat liver PFK-L_2 was a gift from George A. Dunaway, Jr., Southern Illinois University, Springfield, Ill.

Conditions for PFK Inactivation. PFK was routinely treated with FDPase at 23 °C in a buffer composed of 50 mM Tris-HCl (pH 7.5), 2 mM potassium phosphate (pH 7.5), 0.2 mM DTT (Tris–phosphate–DTT buffer). This arbitrary buffer combination was found to give consistently reproducible results for studies of PFK inactivation; thus, reversible inactivation was found upon addition of FDPase and PFK activity still remained stable upon dilution in the absence of FDPase. Dilution of PFK in Tris buffer alone resulted in irreversible denaturation and higher phosphate concentrations reversed inactivation by FDPase. Also, the concentrations of phosphate and DTT could be kept constant in all incubations and assays by adjusting for the concentrations of these components present in different dialyzed PFK preparations.

In each experiment, PFK incubated with FDPase was checked for inactivation, which was usually complete within 5 min. Mixtures were then placed at 4 °C and aliquots taken for assays.

PFK Assay Method. PFK activity was determined using an enzyme-coupled assay to measure the formation of ADP at 30 °C. The rate of NADH disappearance was followed at 340 nm on a Gilford multisample recording spectrophotometer with the full-scale recorder deflection set to 0.200 absorbance units. The final assay volume of 0.50 ml contained 50 mM Tris-HCl (pH 7.5), 3 mM ATP, 5 mM $MgCl_2$, 100 mM KCl, 0.1 mM Fru-6-P, 0.15 mM NADH, 0.5 mM P-enolpyruvate, 3 units of pyruvate kinase, and 4.5 units of lactic dehydrogenase. Pyruvate kinase and lactic dehydrogenase were previously diluted to 600 units/ml and 900 units/ml, respectively, with 10 mM Tris-HCl (pH 7.5) and dialyzed overnight at 4 °C against 500 volumes of the same buffer. The assay reaction was started by adding PFK to the above mixture, and linear rates were obtained within the first minute. The PFK activator mixture used in some experiments consisted of 0.25 M $(NH_4)_2SO_4$, 0.125 M K_2HPO_4, and 10 mM AMP.

Rat liver PFK was assayed under the same conditions as the muscle enzyme except that Tris-HCl buffer was replaced by imidazole buffer (pH 7.5), and 10 mM $(NH_4)_2SO_4$ was included in the cuvette.

Fluorescence Methods. Fluorescence emission measurements were carried out at room temperature in an Aminco-Bowman spectrofluorometer equipped with a xenon arc light source and coupled to an Aminco 10-222 photomultiplier microphotometer. The following slit sizes were used: 4 mm in the two monochromators, 5 mm in the photomultiplier shutter, and 2 mm in the cell holder. Fru-1,6-P_2 was determined using FDPase and a coupled enzyme assay. The fluorescence increase due to the reduction of $NADP^+$ was measured. Excitation and emission wavelengths for NADPH detection were 340 and 465 nm, respectively. Measurements were made in cylindrical fused quartz cells (4-mm inside diameter; capacity 0.5 ml) held in the cell compartment by an appropriate adapter. The assay mixture consisted of 50 mM Tris-HCl (pH 7.5), 150 mM KCl, 0.1 mM EDTA, 0.1 mM $NADP^+$, 5 mM $MgSO_4$, 0.67 unit of Glc-6-P isomerase, 0.14 unit of Glc-6-P dehydrogenase, and 0.27 unit of FDPase in a final volume of 0.220 ml. The auxiliary enzymes were previously diluted and dialyzed against 0.3 M $(NH_4)_2SO_4$. All emission intensities are expressed in arbitrary fluorescence units after correction for the appropriate blanks.

The methods for ANS recrystallization and solution preparation have been published (Aoe et al., 1970). Excitation and emission wavelengths for the PFK–ANS fluorescence studies were 370 and 480 nm, respectively. ANS concentration in all cases was 20 μM.

Preparation of Immobilized Proteins. Bovine serum albumin and goat anti-rabbit liver FDPase were coupled to CNBr-activated Sepharose 4B as previously described (Carlson et al., 1975). FDPase and bovine serum albumin were coupled to Affi-Gel 10 in 0.1 M potassium phosphate buffer (pH 7.5) at 4 °C. In both cases the ratio of protein to Affi-Gel was 10 μg/ml. Although some precipitate was apparent in the coupled gel mixture, the bound FDPase retained enzymatic activity.

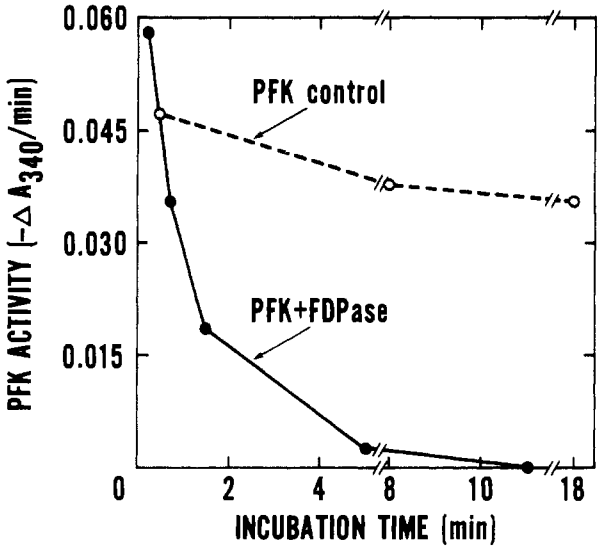

FIGURE 1: Time course of inactivation of PFK by FDPase. PFK control (0.34 mg/ml, (○ - - - ○)) or PFK and FDPase (0.31 mg/ml each (● — ●)) were incubated at 23 °C in Tris-phosphate-DTT buffer and 3-μl aliquots were assayed for activity. Control values are corrected to 0.93 μg of PFK/assay.

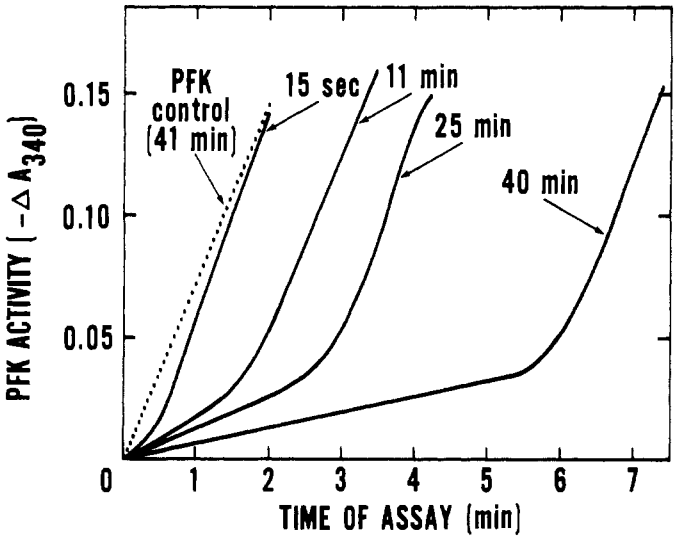

FIGURE 2: Recovery of PFK activity during assays. PFK (0.29 mg/ml) was incubated either alone (· · · ·) or with FDPase (0.32 mg/ml; (—)) in Tris-phosphate-DTT buffer containing 3 mM ATP, 15 mM KCl, and 0.3 mM Mg^{2+}. Aliquots (2 μl) were taken at the indicated times and assayed for activity.

TABLE I: Effect of Dilution Prior to Assay on PFK Activity.

Sample	PFK Act. ($-\Delta A_{340}$ min^{-1} μg^{-1} PFK)	
	−Activators	+Activators[a]
PFK control[b]	0.038	
Tenfold dilution of control[c]	0.041	0.132
PFK + FDPase[d]	0.000	0.124
Tenfold dilution of PFK + FDPase[e]	0.001	0.132

[a] Rate after adding 25 μl of activators to cuvette, corrected for volume change. [b] PFK (0.34 mg/ml) was incubated in Tris-phosphate-DTT buffer for 18 min at 23 °C prior to assay. [c] Aliquots were diluted with Tris-phosphate-DTT buffer and assayed within 1 min. [d] PFK (0.31 mg/ml) + FDPase (0.31 mg/ml) were incubated in Tris-phosphate-DTT buffer for 11 min at 23 °C prior to assay.

TABLE II: Reversal of PFK Inactivation by Mixed Activators.

Sample	μg of PFK/ Assay	PFK Act. ($-\Delta A_{340}$ min^{-1})	
		−Activators	+Activators[a]
PFK control incubation[b]	0.34	0.064	0.141
PFK + FDPase incubation[b]	0.34	0.001	0.169
Direct PFK assay[c]	0.24	0.035	0.110
Assay with 1 μg of FDPase + 20 μM oleate + 100 μM 3-P-glycerate[c]	0.24	0.000	0.096

[a] Rate after adding 25 μl of activators to cuvette, corrected for volume change. [b] PFK (0.34 mg/ml) was incubated 15 min at 23 °C with and without FDPase (0.38 mg/ml) in Tris-phosphate-DTT buffer containing 3 mM ATP. Samples were kept in ice until assayed. [c] Diluted dialyzed PFK added last to cuvette to start assay.

Results

Reversible Inactivation of PFK by FDPase at High Protein Concentration. A rapid and complete loss of rabbit muscle PFK activity was observed when the concentrated enzyme (0.10-0.53 mg/ml) was incubated in Tris-phosphate-DTT buffer containing rabbit liver FDPase (Figure 1). An identical control of PFK incubated without FDPase lost less than 25% of its starting activity in the same time period. The concentration of FDPase necessary to see this inactivation depended upon incubation conditions and the specific activity of PFK. However, inactivation was observed at 30, 23, and 0 °C and did not require the presence of ATP. The inactivation reaction proceeded in the complete absence of added Mg^{2+} or other divalent metal, and hence catalytic activity of FDPase per se was not required. The complete absence of any FDPase activity under these conditions was confirmed directly by assaying aliquots of the incubation mixture.

Table I shows that dilution alone did not reverse the inactivation. When 25 μl of mixed activators ($(NH_4)_2SO_4$, P_i, AMP) was added to each cuvette, both the inactivated PFK and the control assays showed identical high rates. In general, full PFK activity was restored when mixed PFK activators were added to the assay cuvette (Table II). On standing, however, dilute solutions of inactivated PFK slowly lost activity irreversibly.

PFK which was inactivated by relatively low levels of FDPase frequently recovered activity spontaneously during the actual assay (Figure 2). Furthermore, the assay time necessary for this reversal, as well as the actual extent of PFK inactivation, increased as time of incubation before taking assay aliquot was increased. The increasing concentration of Fru-1,6-P_2 that accumulates in the cuvette during the assay may be responsible for this reactivation. In support of this hypothesis, we have found that as little as 1 μM Fru-1,6-P_2 in the assay will reverse more than half of the FDPase inactivation. Also, when 10 μM Fru-1,6-P_2 was included in the incubation mixtures without added Mg^{2+}, nearly all of the FDPase inactivation of PFK was prevented.

ATP (3 mM) was included in several incubation mixtures. This level of ATP helped to protect control PFK activity during

TABLE III: Reversal of PFK Inactivation by Immobilized Anti-Fructose-1,6-bisphosphatase.

Sample	PFK Act. ($-\Delta A_{340}$ min^{-1}) Experiment I (pH 7.5) −Activators	+Activators[a]	Experiment II pH 7.5	pH 8.0[b]
PFK control[c]	0.052	0.147	-	-
PFK + FDPase before columns[d]	0.000	0.177	0.002	0.276
PFK + FDPase after immobilized bovine serum albumin column[e]	0.000	0.075	0.002	0.254
Total recovery of PFK activity[f]		48%		61%
PFK + FDPase after immobilized anti-FDPase column[e]	0.024	0.094	0.086	0.272
Total recovery of PFK activity[f]		58%		66%

[a] Rate after adding 25 μl of activators to the cuvette, corrected for volume change. [b] Assay in 50 mM Tris-HCl (pH 8.0) containing 0.2 mM ATP and 1 mM Fru-6-P. All other assay components remained the same. [c] PFK (0.1 mg/ml) in Tris-phosphate-DTT buffer containing 3 mM ATP and 100 mM KCl, 2 μl/assay. [d] Experiment I: PFK (0.1 mg/ml) was incubated with FDPase (0.21 mg/ml) for 20 min at 23 °C in Tris-phosphate-DTT buffer containing 3 mM ATP and 100 mM KCl, 2 μl/assay. Experiment II: PFK (0.11 mg/ml) was incubated with FDPase (0.26 mg/ml) for 15 min at 23°C in Tris-phosphate-DTT buffer containing 3 mM ATP, 5 μl/assay. [e] Assay on fractions which contained peak PFK activity. Four microliters/assay in experiment I, 15 μl/assay in experiment II. [f] (Sum of total activity in all fractions/total activity applied) 100.

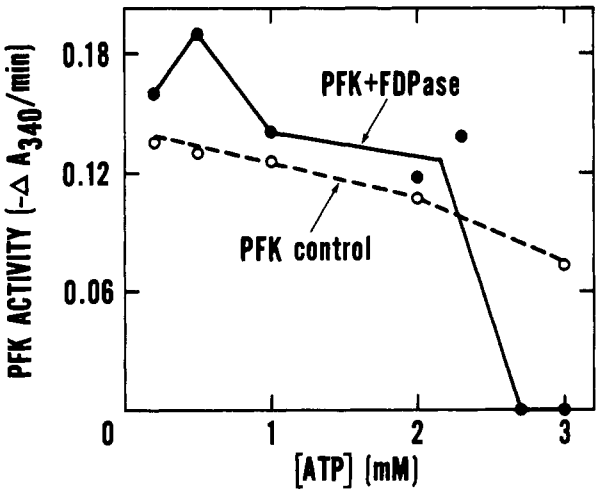

FIGURE 3: Effect of ATP concentration in the assay on activity of PFK inactivated by FDPase. PFK (0.17 mg/ml) and FDPase (0.25 mg/ml) were incubated for 1 min in inactivation buffer containing 3 mM ATP. Aliquots (2 μl) were added to the assay cuvettes containing indicated levels of ATP (● — ●). An aliquot (2 μl) of PFK (0.17 mg/ml) incubated for 1 min alone was assayed at 3 mM ATP. The other control points were calculated from an earlier ATP concentration curve.

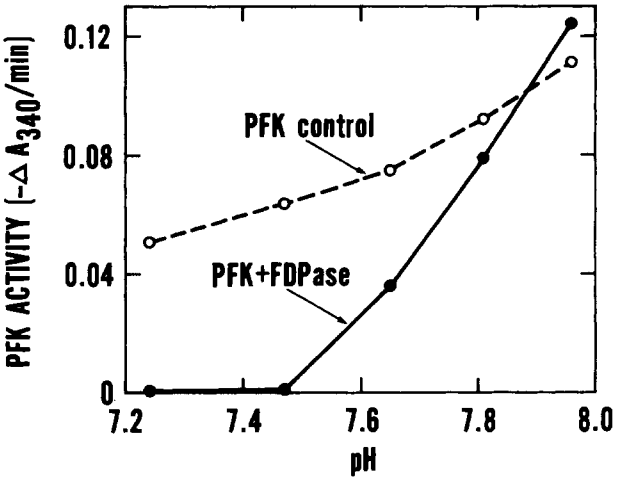

FIGURE 4: Effect of assay pH on activity of FDPase-inactivated PFK. PFK (0.34 mg/ml) was incubated either alone (○ - - - ○) or with FDPase (0.38 mg/ml; ● — ●) for 15 min in inactivation buffer containing 3 mM ATP. Aliquots (1 μl) were assayed at the indicated pH's, which were determined for each complete assay mixture at 30 °C.

incubation. On the other hand, the presence of ATP reduced the amount of FDPase required in the incubation for PFK inactivation. For example, samples from a mixture of PFK and FDPase that contained ATP showed no PFK activity after 4 min of incubation at 30 °C. An identical mixture which did not contain ATP retained greater than 75% PFK activity after 21 min of incubation.

Effect of Assay Conditions. The inactivation of PFK by FDPase was observed when assays were done in Tris-HCl buffer, pH 7.5, containing 3 mM ATP. However, changes in the ATP concentration or assay pH could reverse the inactivation. As may be seen in Figure 3, PFK which had been incubated with FDPase showed activity greater than or equal to the expected control when the ATP concentration in the assay was 2.3 mM or less. Aliquots from the same incubation mixture showed no PFK activity when assayed at higher ATP concentrations. Thus, the activity of PFK that had been incubated with FDPase was very sensitive to changes in ATP concentration in the range of 2–3 mM. The pH of the assay system also had a significant effect on the observed PFK activity. Figure 4 shows that when the assay pH was 7.5 or lower, no PFK activity was observed in the sample mixed with FDPase. At pH values greater than 7.8, control and inactivated PFK show identical rates.

Reactivation of PFK by Removal of FDPase. The results of experiments to test what happened to inactivated PFK after specific removal of FDPase with immobilized anti-FDPase are summarized in Table III. Immobilized bovine serum albumin was used as a control. After inactivation of PFK by FDPase had occurred, 50-μl aliquots of the protein mixture were applied to 0.100- or 0.125-ml columns of Sepharose-immobilized goat anti-rabbit liver FDPase or Sepharose-immobilized bovine serum albumin. The columns were eluted with Tris-phosphate-DTT buffer containing 3 mM ATP and in experiment I, Table III, in addition, 100 mM KCl. Fractions (100 μl) were collected. Peak fractions were assayed at pH 7.5 where FDPase inactivation could be observed and under conditions where total activity could be determined. Total recovery in all fractions based on the starting PFK activity was also determined. It is apparent that the sample from which FDPase was removed had recovered PFK activity at pH 7.5, while the sample which had passed through the bovine serum albumin column remained inactive. In experiment I, the peak PFK fraction from the bovine serum albumin column contained 87% of the initial FDPase activity, while the corresponding fraction from the anti-FDPase column had no FDPase activity. Full PFK activity

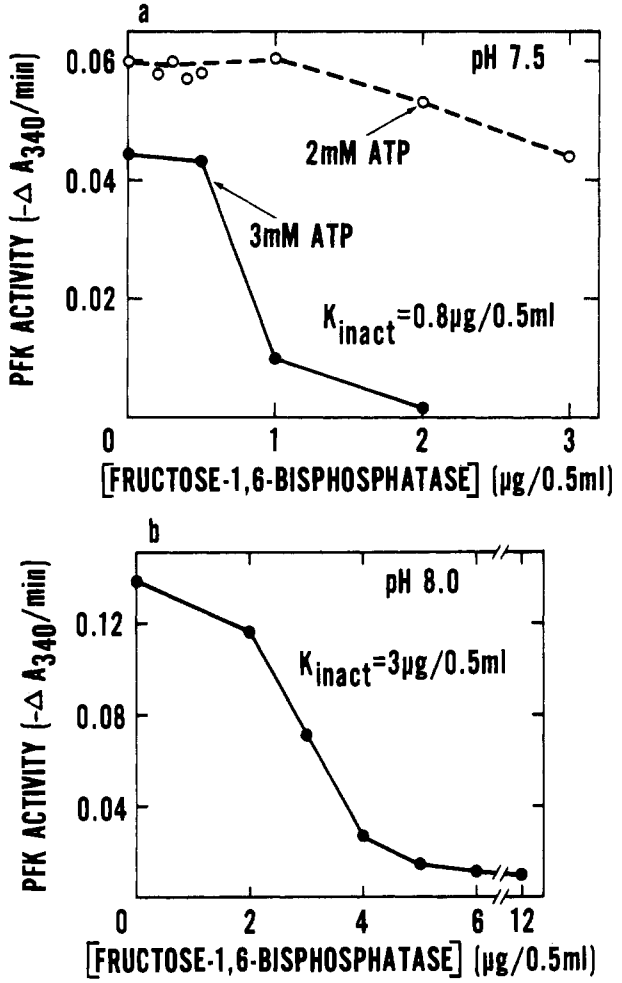

FIGURE 5: Inactivation of PFK by FDPase in the assay at (a) pH 7.5 in the presence of 2 and 3 mM ATP and (b) at pH 8.0 in the presence of 3 mM ATP. Amounts of PFK in assays were (a) 0.27 µg and (b) 0.25 µg, respectively.

could be recovered when similar control PFK incubation mixtures were passed through either Sepharose or bovine serum albumin-Sepharose columns. However, a time-dependent irreversible denaturation, which was observed after dilution of inactivated PFK, could account for the variation in recovery of total PFK activity in these experiments.

Specificity of PFK Inactivation by FDPase. Several other purified proteins were incubated with PFK to confirm the specificity of the inactivation process. Under conditions where "neutral" FDPase (0.4–0.5 mg/ml) produced >90% inactivation of PFK (0.2–0.3 mg/ml) in 10–15 min, neither aldolase (3 mg/ml), bovine serum albumin (3.4 mg/ml), DNase (1.6 mg/ml), nor "alkaline" FDPase (0.43 mg/ml) had any effect on PFK activity.

Reversible PFK Inactivation by FDPase at Low Protein Concentrations. In order to make certain that the PFK inactivation was not merely an effect of FDPase in the assay cuvette, aliquots of PFK were assayed under identical conditions except that one cuvette contained FDPase at a concentration equivalent to that in an aliquot taken from the inactivated mixture. This low level of enzyme in the cuvette had no effect on PFK activity. However, higher levels of FDPase in the assay were able to inactivate PFK that was added directly to the cuvette. Figure 5a shows that the amount of FDPase required for maximal PFK inactivation was about eight times the weight of PFK in the presence of 3 mM ATP. The inactivation was much less apparent at 2 mM ATP. Addition of 0.1 mM EDTA, which increases FDPase activity at least tenfold at this pH (Pogell et al., 1968), had no significant effect on the level of FDPase needed for inactivation.

Figure 5b shows that, compared to pH 7.5, a threefold greater amount of FDPase was required in the cuvette at pH 8.0 to cause 50% PFK inactivation. At even higher levels of FDPase, greater than 90% PFK inactivation was observed at pH 8.0 in the presence of 3 mM ATP. As before, PFK activity was completely restored when 25 µl of mixed PFK activators were added to the cuvette.

Reversible Inactivation of Rat Liver PFK by Rabbit Liver FDPase. Most of our studies were carried out with rabbit muscle PFK and liver FDPase. However, similar results with enzymes from the same organ seemed necessary before inference of any physiological significance was justified. We therefore carried out a few experiments with rat liver PFK to confirm that similar effects are found with PFK and FDPase from the same organ (Sankaran et al., 1975b). Rat liver PFK-L_2 (0.18 mg/ml, 50 units/mg of protein) incubated before assay at 30 °C with rabbit liver FDPase (0.42 mg/ml) in the same buffer used with muscle PFK was 90% inactivated within 30 s. The rat liver enzyme was also found to be very sensitive to FDPase inactivation when tested directly in the assay cuvette. As little as 0.4 µg of FDPase lowered PFK activity of 1.8 µg of liver PFK to less than 10% of the control rate. In all cases, rates equal to or greater than those of controls were observed after addition of mixed activators.

Uyeda and Luby (1974) found that interaction of PFK and FDPase from homologous or different organs gave similar results. These results were not too surprising, since immunological studies suggest that rabbit muscle and liver PFKs have considerable similarity in structure. Thus, guinea pig anti-rabbit muscle PFK partially precipitated rabbit liver PFK (Tsai and Kemp, 1973). In our laboratory, we have observed cross-reactivity among PFKs in crude extracts of rabbit liver and muscle and pure rabbit muscle PFK using goat anti-rabbit muscle PFK in the Ouchterlony double immunodiffusion technique. Also, Sepharose-immobilized anti-muscle PFK removed this cross-reacting antigen from dialyzed liver supernatant (Carlson et al., 1975).

Determination of Fru-1,6-P_2 Bound to PFK. FDPase could have been inactivating PFK by removing tightly bound Fru-1,6-P_2 that was essential for activity (El-Badry et al., 1973). However, examination of our PFK stocks indicated that essentially no Fru-1,6-P_2 was present. A sensitive fluorometric assay with purified FDPase was used (see Materials and Methods) which gave linear increases in fluorescence with increasing concentrations of Fru-1,6-P_2 (24 arbitrary units/nmol at 465 nm). In all cases, the amount of Fru-1,6-P_2 present in dialyzed or undialyzed PFK (0.8–1.4 nmol) averaged less than 0.1 mol/320 000 g. If Fru-1,6-P_2 was later added to an assay cuvette containing PFK, the expected increase in fluorescence was observed, indicating that PFK did not bind Fru-1,6-P_2 in such a way that it was not accessible to FDPase. Also, after extraction of crystalline PFK (1.7 nmol) with 4 M $HClO_4$, neutralization with KOH, and centrifugation, no Fru-1,6-P_2 was found in the supernatant.

Attempts to Demonstrate Direct Protein–Protein Interaction. We have tried numerous physical techniques to obtain direct evidence for some weak interaction between PFK and FDPase, all of which were unsuccessful. No changes were observed (1) in the position of either enzyme activity after sucrose density gradient centrifugation, (2) in elution patterns of PFK from Sephadex G-200 columns, (3) in elution profiles

and total recovery of PFK activity from Affi-Gel 10-immobilized bovine serum albumin and FDPase, and (4) in differences in the penetration of FDPase alone or with PFK into Sephadex G-150 by the procedure of Fahien and Smith (1974).

Changes in the fluorescence of the PFK–ANS complex caused by FDPase were also not adequate to demonstrate a specific interaction. In agreement with the results of Uyeda and Luby (1974), we consistently found that FDPase decreased the fluorescence intensity of PFK–ANS mixtures, both in the presence and absence of ATP. Also, the fluorescence emission was lowered by ATP alone. However, the maximum change in fluorescence did not correlate with the amount of FDPase needed to inactivate PFK. Furthermore, other proteins including DNase and aldolase were able to decrease the fluorescence of our dialyzed commercial PFK preparations. In all cases, the fluorescence reading of a cuvette containing everything except PFK was subtracted from the experimental reading in order to obtain the fluorescence due to PFK.

Synergistic Effects of Oleate, 3-P-glycerate, and FDPase on PFK Inactivation. Both oleate and 3-P-glycerate are known positive effectors of liver FDPase. Oleate, in the presence of liver cytosol proteins, activates FDPase at pH 7.3 to the same extent as EDTA (Carlson et al., 1975). On the other hand, 3-P-glycerate prevents and reverses inactivation of FDPase by ATP or ADP, but does not increase catalytic activity by itself (Pogell et al., 1971). Muscle and brain PFKs are inhibited by 3-P-glycerate, and synergistic inhibition of muscle PFK is found with 3-P-glycerate plus FDPase (Uyeda and Luby, 1974). Uyeda and co-workers (Ramadoss et al., 1976) have also found that an irreversible inactivation of muscle PFK occurs when the enzyme was first incubated at 35 °C in the presence of various fatty acids. A tight binding of [^{3}H]oleate to PFK was observed, and inactive enzyme was completely dissociated into dimers. These observations prompted us to study the combined action of oleate, 3-P-glycerate, and FDPase on PFK. Under our conditions, where PFK was always added last to assay cuvettes containing the various effectors, either oleate or 3-P-glycerate individually was only partially inhibitory. Oleate (20–50 μM) or 3-P-glycerate (100 μM) produced only a 25% drop in PFK activity. Even at higher concentrations (100 μM oleate or 1 mM 3-P-glycerate) only 42–47% inhibition was found. In general, FDPase produced a greater decrease in PFK activity when tested in the presence of either oleate or 3-P-glycerate, but the greatest synergism was always seen in the presence of all three effectors. As seen in Figure 6, whereas 20 μM oleate plus 100 μM 3-P-glycerate, or FDPase alone, had little or no effect, the three effectors together acted synergistically to produce greater than 90% inactivation of PFK. When the concentration of Fru-6-P in the assay was 0.3 mM or greater, PFK inactivation by these effectors was prevented.

Incubation of PFK (0.24 μg) for 3 min at 30 °C with 40 μM oleate plus 100 μM 3-P-glycerate in the assay mixture minus Fru-6-P also caused inactivation. Several minutes after the assay was initiated by addition of Fru-6-P, PFK activity recovered spontaneously (similar to results shown in Figure 2). In contrast to the irreversible PFK inactivation by oleate (Ramadoss et al., 1976), full activity could be restored in all our synergism studies by the addition of mixed activators (Table II, Figure 6).

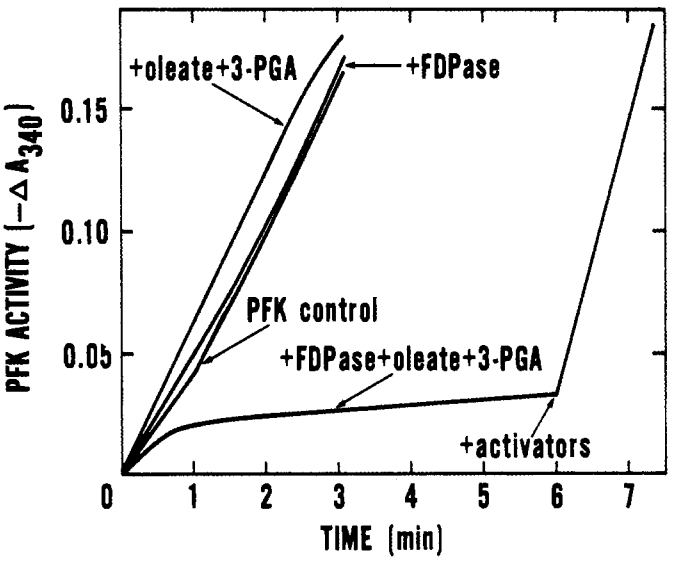

FIGURE 6: Synergistic inactivation of PFK by oleate, 3-P-glycerate, and FDPase. Amounts present in assays: PFK, 0.24 μg; FDPase, 1 μg; oleate, 20 μM; 3-P-glycerate, 100 μM, where indicated. Mixed activators were added at the time indicated.

Discussion

Our results show that under the appropriate conditions PFK is specifically and reversibly inactivated by FDPase. This effect was observed at all PFK concentrations studied, provided that the FDPase level was adequate. The minimum size of PFK necessary for high catalytic activity is assumed to be the tetramer (320 000) (Aaronson and Frieden, 1972; Pavelich and Hammes, 1973). In the presence of ATP, pfk activity is inhibited (Uyeda and Racker, 1965), the tetrameric form is stabilized (Lad et al., 1973), and the fluorescence emission of the PFK–ANS complex is decreased (Bloxham, 1973; Uyeda and Luby, 1974). All of these observations support a conformational change of the tetramer to a low activity form. Our present experiments also show that both ATP (Figures 3, 5) and oleate plus 3-P-glycerate (Figure 6) act synergistically with FDPase to induce the formation of PFK with much lower specific activity. Incubation of PFK with high concentrations of FDPase alone can bring about this conversion in the absence of ATP. However, reactivation occurs under our assay conditions unless at least 2.7 mM ATP is present. We believe the rapid restoration of nearly full activity by activators of PFK is more consistent with a conformational change of the tetramer, although formation of inactive dimer under these conditions cannot be eliminated. Lad et al. (1973) have estimated that the half-time for conversion of dimers, formed in the presence of citrate or by dilution, to tetramers is <5 min at 5 °C. Upon prolonged incubation or dilution of PFK, some activity is lost irreversibly, probably by the formation of inactive dimer (Pavelich and Hammes, 1973).

Mechanism of PFK Inactivation by FDPase. Two explanations have been suggested for the molecular mechanism of PFK inactivation by FDPase: (1) Fru-1,6-P_2 tightly bound on PFK is hydrolyzed by FDPase, resulting in the conversion of PFK to a less active form (El-Badry et al., 1973). These experiments were done with pure sheep heart PFK, where binding of Fru-1,6-P_2 at both catalytic and allosteric sites has been demonstrated (Setlow and Mansour, 1972). We have observed that low levels of Fru-1,6-P_2 both prevent and reverse inactivation of PFK by FDPase. (2) A weak protein–protein interaction between the two enzymes results in the formation of PFK with very low activity (Uyeda and Luby, 1974). These results were carried out with purified rabbit muscle and chicken liver PFKs and purified rabbit muscle and chicken liver FDPases, and included the only direct physical evidence for

protein–protein interaction, namely, the specific quenching of PFK–ANS fluorescence by addition of FDPase. A high degree of specificity for FDPase was found. Even slight modifications of the enzyme, such as formation of the homocystine- or CoA-treated derivatives which have three to five times higher FDPase activity (Nakashima et al., 1970), completely eliminated the effects on PFK.

Our present experiments lend strong support to the second argument. Thus, it is difficult to explain the following observations by any other mechanism than some sort of weak protein–protein interaction: (a) PFK inactivation by FDPase occurs in the complete absence of Mg^{2+}, which is required for FDPase catalysis. We were careful to confirm the complete absence of FDPase activity under these conditions. Furthermore, aliquots taken from the same incubation mixture at different times showed progressively decreasing PFK activity, supporting the argument that the change in PFK had occurred prior to assay. (b) Direct analysis of the preparations of muscle PFK used in our studies showed the presence of <0.1 mol of Fru-1,6-P_2/mol of tetrameric PFK. (c) Of the proteins tested under these conditions, only FDPase was able to bring about PFK inactivation. In particular, aldolase had no effect when incubated with PFK before addition to the assay. (d) Increased amounts of FDPase caused PFK inactivation at pH 8, where the effects of allosteric regulators of PFK are greatly reduced. Under our assay conditions at pH 8, addition of mixed activators only doubled the observed rate.

However, since the evidence for direct protein–protein interaction between PFK and FDPase is based primarily on measurements of enzyme activities, and in the absence of direct physical evidence other than the reported specific decrease in PFK–ANS fluorescence after addition of FDPase (Uyeda and Luby, 1974), we feel that no final conclusion is possible on the actual mechanism of PFK inactivation. In fact, it is highly possible that both phenomena occur.

Physiological Considerations. A recent in vivo study from Lardy's laboratory (Clark et al., 1974) on the relative activities of PFK and FDPase in rat liver suggests a close interrelationship between these two enzyme activities which results in a precise control of the flux of metabolites through the "futile" cycle. From their data, we have estimated that the expressed activities of PFK and FDPase in rat liver are never more than 7–9% of the calculated maximum. These observations are consistent with the observed interaction of PFK and FDPase in vitro.

Thus, PFK and FDPase must be under tight physiological control to regulate a metabolic flux through Fru-6-P and Fru-1,6-P_2 either in favor of glycolysis or gluconeogenesis. In vitro studies of regulator effects have led to some understanding of how this may be accomplished in vivo. Whatever the molecular mechanism may be, our present results show that at very low relative molar ratios, homogeneous "neutral" rabbit liver FDPase can inactivate both rabbit muscle and rat liver PFKs. Thus, incubation of 2.3–5.0 mol of FDPase with 1 mol of muscle PFK resulted in complete inactivation (Figure 1). Under assay conditions as little as 1 mol of FDPase inactivated 1 mol of rat liver PFK-L_2. Since little or no inactivation was observed at lower ratios, it appears that at least 1 mol of FDPase/tetramer of PFK is required for inactivation. The inactivation was still found at high protein concentrations, approaching those found in vivo. With sheep heart PFK, inactivation by aldolase occurred only at PFK concentrations below 1 μg/ml (El-Badry et al., 1973).

The existence of a PFK–FDPase interaction provides another mechanism which could impart a much greater degree of refinement to the intracellular control of substrate cycling. Evidence from in vivo or liver perfusion studies indicates that there is not a sufficient variation in tissue concentrations of ATP, ADP, or AMP to bring about significant alterations in the two enzyme activities (Clark et al., 1974; Tarnowski and Seemann, 1967; Exton and Park, 1969; Burch et al., 1970; Hems et al., 1966). Under conditions that stimulate gluconeogenesis (starvation, glucagon administration, and diabetes), the known rise in the concentration of 3-P-glycerate (Exton and Park, 1969) and possibly free fatty acids (Shafrir and Ruderman, 1974) would favor increased FDPase activity and, in the presence of the high intracellular levels of ATP (>3 μmol/g wet weight), decreased PFK activity. Refeeding or a lowered glucagon level would result in a drop in 3-P-glycerate concentrations that would allow inactivation of FDPase by ATP and increased activity of PFK. In this regard, the peptide stabilizing factor for liver PFK recently described by Dunaway and Segal (1974) may also have a role in regulation of the "futile" cycle. The concentration of this factor is low during starvation or diabetes, and rises upon glucose refeeding or insulin administration. We have recently shown that this factor prevents inactivation of both muscle and liver PFKs by FDPase, and also lowers the activity of liver FDPase in the presence of oleate plus cytosol proteins (Sankaran et al., 1975b).

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Cytochrome *c* Induced Lateral Phase Separation in a Diphosphatidylglycerol–Steroid Spin-Label Model Membrane†

G. Bruce Birrell and O. Hayes Griffith*

ABSTRACT: The extrinsic membrane protein cytochrome *c* binds to lipid mixtures containing negatively charged phospholipids such as diphosphatidylglycerol (DPG). In this study the effect of cytochrome *c* on the lipid distribution in a DPG–steroid spin-label (3-doxyl-5α-cholestane) model membrane system is examined. The electron spin resonance (ESR) line-shape changes indicate that cytochrome *c* induces lateral phase separation at room temperature. The resulting two-dimensional lipid distribution is nonrandom, consisting of clusters of phospholipids bound to cytochrome *c* and patches of steroid spin-label molecules. Phase separations are also observed in the three-component system: DPG, phosphatidylcholine, and 3-doxyl-5α-cholestane.

Lipid-protein interactions in biological membranes can be examined by observing differences in mobility of lipid spin-labels (Griffith and Jost, 1976). In the large protein complex cytochrome oxidase (cytochrome a, a_3), the terminal member of the mitochondrial electron transport chain, a fraction of the lipid is observed to be immobilized (Jost et al., 1973). This is consistent with the idea that cytochrome oxidase and other integral proteins penetrate deeply into or completely through the phospholipid bilayer, creating a hydrophobic lipid–protein interface. In contrast to the behavior of integral proteins, extrinsic proteins such as cytochrome *c* are easily released from membranes by dilute salt solutions (Jacobs and Sanadi, 1960). In its normal functional role, the small protein cytochrome *c* transfers electrons from one large integral protein complex (cytochrome b–c_1) to another (cytochrome oxidase), but it is also known to bind to bilayers containing negatively charged lipids (Green and Fleischer, 1963; Gulik-Krzywicki et al., 1969; Nicholls, 1974; Kimelberg et al., 1970; Vanderkooi et al., 1973), and specific lipid–protein interactions may be important to the function of cytochrome *c*. In a previous study, the motion of fatty acid spin-labels before and after addition of cytochrome *c* was examined in bilayers of the negatively charged phospholipid diphosphatidylglycerol (DPG, cardiolipin) and phosphatidylcholine (PC, lecithin).[1] Any effects of cytochrome *c* were small compared to the marked immobilization by integral proteins (Van and Griffith, 1975). These data are consistent with electrostatic binding of cytochrome *c*.

In this paper we examine whether cytochrome *c* can influence the local arrangement of lipids in the plane of the mem-

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[1] Abbreviations used are: DPG, diphosphatidylglycerol; PC, phosphatidylcholine; ESR, electron spin resonance.