Fish Muscle Phosphofructokinase: Influences of Protein Concentration on Enzyme Kinetic Behaviour

JEFFREY Y. SU,* KENNETH B. STOREY†

Institute of Biochemistry and Department of Chemistry, Carleton University, Ottawa, Ontario, Canada K1S 5B6

Phosphofructokinase (PFK) is regulated by multiple mechanisms, one of these being changes in the polymerization state of the enzyme which is influenced by enzyme concentration. To better understand the regulation of PFK in vivo, the present study monitored the effects of changing enzyme concentration and of protein crowding on enzyme activity and properties. Purified PFK from white muscle of rainbow trout (Oncorhynchus mykiss) was characterized under assay conditions that simulated the physiological state of resting muscle [pH 7.2, 7.5 mM MgATP, 0.2 mM fructose-6-phosphate (F6P)], and with experimental manipulation of enzyme concentration in two ways: (a) increasing the amount of PFK (0.1 or 0.6 μg/ml) added, and (b) crowding the enzyme by the addition of polyethylene glycol (PEG) (or other agents) to the assay mixture. A marked decrease in ATP inhibition of the enzyme was observed when PFK was studied in buffer containing PEG, as well as when assays were carried out with the purified enzyme at a concentration of 0.6 μg/ml, as compared with a 6-fold diluted enzyme. Enzyme concentration also altered the F6P saturation curve, S0.5 values for F6P decreasing when PFK concentration was increased or crowding reagents were added. The inclusion of PEG in the assay mixture shifted the pH profile of PFK leftward with much higher enzyme activity observed as compared to that in the absence of PEG, especially at low pH values. These results provide evidence that the allosteric behaviour of fish muscle PFK is affected by enzyme concentration and support the proposal that the more aggregated forms of the enzyme have higher affinity for fructose 6-phosphate and are less inhibited by Mg.ATP.

Keywords: Polyethylene glycol crowding of enzymes Glycolytic regulation in fish muscle Oncorhynchus mykiss Enzyme concentration Allosteric properties

INTRODUCTION

Phosphofructokinase (PFK, ATP:D-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.11) plays an important role in the regulation of glycolysis and, hence, the regulatory properties of PFK have been the focus of intensive research. In animal tissues PFK is a multimeric enzyme whose activity responds to a variety of metabolic signals (Uyeda, 1979; Sols et al., 1981). PFK may exist in several different states in vivo, depending on the degree of subunit phosphorylation or the extent of binding with structural elements of the cell, both of which also regulate the kinetic activity of the enzyme (Storey, 1987; Brooks and Storey, 1988). In addition, the enzyme from different sources has been shown to polymerize, adding another dimension to the regulation of PFK activity. The polymerization state of PFK and hence, the kinetic properties of the enzyme, are affected by enzyme concentration (Hofer and Krystek, 1975; Reinhart and Lardy, 1980; Reinhart, 1980; Bosca et al., 1985). Aragon et al. (1980) observed that the regulatory behavior of PFK in situ was markedly different from that observed in vitro. This finding may be partly due to a dependence of PFK polymerization on enzyme concentration as it is well known that
Many intracellular enzymes are present in vitro at much higher concentrations than are used for in vitro assays (Sols and Marco, 1979). Therefore, it is important, if possible, to study the kinetic behavior of PFK at physiological concentrations of the enzyme.

Various procedures have recently been used in attempts to allow kinetic analysis of PFK at physiological concentrations of enzyme. These include the cell permeabilization method, studies of purified enzyme at concentrations close to the in vitro range, and the addition of polyethylene glycol (PEG) (Aragon and Sols, 1991). PEG has been used in an attempt to reproduce the high viscosity of cellular fluids (Srivastava and Bernhard, 1987) as well as to "crowd" the enzyme. The homogeneous and heterogeneous enzyme-enzyme interactions that may occur at the high protein concentrations prevailing in vivo can be simulated in vitro by the use of PEG (Srere, 1987). PEG apparently acts through a "steric exclusion" mechanism in which the long chains of PEG confine the protein to relatively small pockets of solvent, thus increasing the local enzyme concentration and favouring protein aggregation (Lee and Lee, 1987). PEG has been widely used to study the potential influence of enzyme concentration on the activity of PFK (Reinhart, 1980; Aragon and Sanchez, 1985; Bosca et al., 1985) and other enzymes (Medina et al., 1985; Mortberg and Neujahr, 1987).

In this paper, the effects of enzyme concentration on the allosteric behavior of fish muscle PFK were analysed by increasing PFK concentration or adding a "crowding" agent such as PEG. In addition, the allosteric influences of various cellular metabolites on the kinetic behavior of the enzyme probably have at least as great a role in the ultimate determination of enzyme activity in the cell as do the nonallosteric kinetic constants (Su and Storey, 1994). Consequently, the present study was carried out using conditions of substrate levels (7.5 mM Mg·ATP, 0.2 mM F6P) and pH (7.2) that resemble the physiological situation in vivo in resting trout white skeletal muscle. The results indicate that the kinetic and regulatory properties of purified white muscle PFK under such conditions are highly dependent on enzyme concentration. These results provide evidence that supports the contention that the allosteric regulation of fish muscle PFK depends on its concentration in vivo.

**METHODS AND MATERIALS**

ATP, ADP and NADH were from Boehringer Mannheim, Montreal. Glycerol was from Mallinckrodt Inc. Other chemicals, biochemicals and the coupling enzymes were from Sigma Chemical Co. Trout muscle PFK was prepared according to Su and Storey (1992). The enzyme preparation had a final specific activity of approx. 70 U/mg protein assayed at pH 7.2 and 22 °C and was shown to be homogeneous by SDS-polyacrylamide gel electrophoresis (Su and Storey, 1992). The purified enzyme was stored at −20 °C in buffer containing 40% (v/v) glycerol and 20 mM phosphate (pH 7.5). Before use, PFK was dialyzed against 20 mM imidazole buffer containing 10 mM 2-mercaptoethanol (pH 7.2) at 4 °C to remove inorganic phosphate and glycerol in the storage buffer.

PFK was assayed by following NADH oxidation on a Gilford 240 spectrophotometer at 340 nm using plastic cuvettes with standard assay conditions at 22 °C as follows: 20 mM imidazole buffer, pH 7.2, 0.15 mM NADH, 5 mM MgCl2, 0.2 mM F6P, 7.5 mM ATP, 2 U aldolase, 2 U triose-phosphate isomerase, and 1 U glycerol-3-phosphate dehydrogenase in a total volume of 1 ml. All ATP stock solutions contained added MgCl2 in a 1:1 molar ratio. The pH of PEG stock solutions was adjusted to 7.2 before use except for pH curves. Purified trout muscle PFK was added to initiate the reaction. Coupling enzymes were dialyzed against a 500-fold excess of buffer (20 mM imidazole-HCl, pH 7.2, 10 mM 2-mercaptoethanol) at 4 °C for >12 hr with at least two changes of buffer to remove ammonium sulfate which was found to activate PFK. Alternatively, the ammonium sulfate in the coupling enzymes was removed by two serial centrifugations through 5 ml columns of Sephadex G-25 (Helmerhorst and Stokes, 1980).

Protein concentration was determined by the Bio-Rad protein assay kit based on the Coomassie Blue G-250 dye-binding method with bovine gamma globulin as the standard (Bradford, 1976).

Substrate affinity constants (S0.5) and Hill coefficients (nH) were determined by fitting the data to the Hill equation using a nonlinear least-squares regression computer program (Brooks, 1992). Inhibitor constants (I50) were obtained from plots of reaction velocity vs inhibitor concentration. Results are presented...
Protein concentration influences PFK kinetics

as means ± SEM for at least $n = 3$ determinations on separate preparations of the purified enzyme from different fish. The Student's $t$-test was used to test for significant differences between values.

RESULTS

The activity of trout white skeletal muscle PFK was significantly influenced by enzyme concentration. Enzyme specific activity (U/mg protein) increased with increasing amounts of PFK added to the assay mixture as shown in Fig. 1. Furthermore, the addition of 10% (w/v) PEG which increases local enzyme concentration resulted in a further increase in enzyme specific activity over the full range of PFK concentrations tested. This concentration dependence of PFK activity has been reported previously for the enzyme from other sources (Reinhart, 1980; Aragon et al., 1980; Bosca et al., 1985; Ovadi et al., 1986). It is believed that the decrease in specific activity at low enzyme protein concentrations is due to dissociation of the enzyme tetramer.

The influences of enzyme concentration or the addition of PEG on the kinetic behavior of trout muscle PFK are shown in Figs 2 and 3. The kinetic parameters of the enzyme under these conditions are summarized in Table 1. When PFK activity was assayed at a dilute concentration, as is common in in vitro studies, a sigmoidal fructose-6-phosphate (F6P) saturation curve ($n_H > 1$) was found. Based on enzyme purification data (Su and Storey, 1992), the in vitro concentration of PFK in white muscle of rainbow trout was estimated to be 0.38 mg/ml, a value close to those reported previously (Ling et al., 1965; Ramados et al., 1976). Thus, both PFK concentrations employed presently, 0.1 and 0.6 μg/ml, were below the physiological level. Nevertheless, an increase in the enzyme affinity for F6P was observed by increasing PFK concentration or adding "crowding reagents" such as PEG (Fig. 2 and Tables 1, 2). As shown in Fig. 2, the typical sigmoidal F6P saturation curve was strongly shifted to the left when PFK was assayed at a higher enzyme concentration or with the inclusion of PEG. The $S_{50}$ value decreased by one order of magnitude when PFK was used at 0.6 μg/ml in the presence of 10% (v/v) PEG as compared to the assay carried out at 6-fold diluted concentration of enzyme in the absence of PEG (Table 1). Similar results were obtained by using other "crowding" agents which are believed to increase local enzyme concentration. Dimethylsulfoxide and glycerol also caused an increase in the activity of PFK proportional to the increase in concentration of the "crowding" agents (Table 2). Similar to the situation with
added PEG, the affinity for F6P was uniformly increased by adding these compounds as evidenced by the changes in the ratio of enzyme velocity (v) at suboptimal vs infinite F6P concentration (Table 2). Thus, compared with the v/Vmax activity ratio without addition, 20% DMSO increased the ratio by 8.8-fold and 40% glycerol increased the ratio by 4.2-fold whereas 20% PEG increased the ratio by 8-fold.

PFK activity is highly sensitive to changes in the concentrations of its substrate and negative allosteric effector, ATP (Uyeda, 1979). The enhancement in apparent affinity for F6P observed at high enzyme concentration was also accompanied by a change in the ATP inhibition. Figure 3 shows the effects of changes in enzyme concentration or the addition of PEG on the observed ATP inhibition of the enzyme. ATP inhibition was strongly reduced when the enzyme was assayed at the higher PFK concentration and ATP inhibition was greatly reduced at both PFK concentrations in the presence of 10% PEG. As Table 1 shows, the I50 value for Mg.ATP increased by nearly 5-fold when PFK concentration was increased from 0.1 to 0.6 μg/ml. I50 Mg.ATP was 9-fold higher in the presence vs absence of 10% PEG for PFK assayed at 0.1 μg/ml concentration and 3.4-fold higher for the comparison at 0.6 μg/ml enzyme concentration. It is obvious that the influence of PEG is more profound when PFK concentration is low.

PFK activity was also highly sensitive to changes in pH and physiological pH change in muscle appears to be one important factor in enzyme control in vitro. Intracellular pH in trout muscle falls during muscle work correlated with lactic acid accumulation due to activated glycolysis (Milligan and Wood, 1987). The response of PFK activity to pH change was also found to be dependent on enzyme concentration. Figure 4 shows the effect of the addition of 10% PEG on the pH profile of the enzyme. Inclusion of PEG in the assay mixture shifted the pH curve to the left; for example, in the absence of PEG, PFK activity at pH 7.0 was less than 5% of the maximum but in the presence of PEG, 60% of maximum activity was retained. Addition of the other "crowding" agents or a space-filling macromolecule (bovine serum albumin) gave similar results (data not shown). The effects of PEG were very similar to those of known allosteric activators of the enzyme in left-shifting the pH profile of PFK; for example, the effect of AMP is shown for comparison in Fig.4.

**DISCUSSION**

In the present study, an inert polymer, PEG, was employed to artificially crowd enzyme molecules in order to study PFK kinetics at high protein concentrations. PEG, similar to other randomly coiled water-soluble polymers, interacts unfavorably with proteins and is repelled (Lee and Lee, 1987). The two species (polymer and protein) tend to remove the other from the water phase in which both are present and this favors the formation of phases in which the species are separate in order to minimize the destabilization of polymer–protein interaction.
Protein concentration influences PFK kinetics

Table 1. Effect of enzyme concentration on the kinetic properties of trout muscle PFK

<table>
<thead>
<tr>
<th>[PFK] (μg/ml)</th>
<th>[PEG] (% w/v)</th>
<th>S₅₀ for F6P (mM)</th>
<th>I₅₀ for ATP (mM)</th>
<th>n</th>
<th>Vₒₒ (relative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0</td>
<td>3.54 ± 0.17</td>
<td>1.46 ± 0.07</td>
<td>1.00 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td>0</td>
<td>0.73 ± 0.02</td>
<td>1.91 ± 0.04</td>
<td>1.26 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>10</td>
<td>0.75 ± 0.01</td>
<td>2.39 ± 0.07</td>
<td>1.93 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td>10</td>
<td>0.37 ± 0.02</td>
<td>2.98 ± 0.06</td>
<td>2.04 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>0</td>
<td>0.02 ± 0.01</td>
<td>1.00 ± 0.03</td>
<td>1.00 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td>0</td>
<td>0.94 ± 0.03</td>
<td>4.42 ± 0.25</td>
<td>4.35 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>10</td>
<td>1.87 ± 0.05</td>
<td>1.73 ± 0.06</td>
<td>2.31 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td>10</td>
<td>3.23 ± 0.02</td>
<td>2.12 ± 0.06</td>
<td>4.33 ± 0.10</td>
<td></td>
</tr>
</tbody>
</table>

PFK was assayed at 22 °C in 20 mM imidazole buffer, pH 7.2 in the presence of (1) 7.5 mM ATP or (2) 0.2 mM F6P. Results are means ± SEM, n = 3. *Significantly different from the corresponding value determined at 0.6 μg/ml enzyme concentration using one-way analysis of variance and the Student-Newman-Keuls test (2-tailed), P < 0.05: **significantly different from the corresponding value without PEG.

Table 2. Effect of "crowding" reagents on the activity of trout muscle PFK

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration (% w/v)</th>
<th>Relative Activity</th>
<th>Vₒₒ (relative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>1.00 ± 0.04</td>
<td>0.126</td>
</tr>
<tr>
<td>PEG</td>
<td>5 (w v)</td>
<td>2.61 ± 0.21</td>
<td>0.316</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4.74 ± 0.63</td>
<td>0.574</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>8.22 ± 0.80</td>
<td>0.994</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>8.26 ± 0.83</td>
<td>1.000</td>
</tr>
<tr>
<td>DMSO</td>
<td>2.5 (w v)</td>
<td>1.96</td>
<td>0.237</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>3.74 ± 0.11</td>
<td>0.453</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>8.54 ± 0.11</td>
<td>1.034</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>9.13</td>
<td>1.015</td>
</tr>
<tr>
<td>Glycerol</td>
<td>5 (w v)</td>
<td>1.46 ± 0.07</td>
<td>0.174</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.61 ± 0.09</td>
<td>0.315</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>3.59 ± 0.04</td>
<td>0.434</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>3.96 ± 0.09</td>
<td>0.482</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>4.41 ± 0.07</td>
<td>0.534</td>
</tr>
</tbody>
</table>

Purified trout muscle PFK was used at a concentration of 0.6 μg/ml. The concentrations of F6P and ATP were 0.2 and 7.5 mM, respectively. Vₒₒ was the calculated rate obtained in the presence of 7.5 mM ATP and infinite concentration of F6P. Results are means ± SEM, based on at least three determinations: where no SEM is shown for relative activity, n = 1.

Knoll and Hermans, 1983]. A large number of compounds with a variety of chemical structures, such as glycerol (Gekko and Timasheff, 1981) and PEG (Arakawa and Timasheff, 1985; Lee and Lee, 1987) are known to be preferentially excluded from the immediate domain of proteins. As a result of the preferential exclusion of solute from the protein surface, the local protein concentration is increased (Miekka and Ingham, 1978; Lee and Lee, 1987).

Middaugh et al. (1979) have reported that radiolabeled PEG was quantitatively excluded from protein aggregates, which suggests that the observed changes in enzyme properties upon the addition of PEG does not result from interactions between the enzyme and the polymer. In the present study, both a direct increase in enzyme concentration or an indirect increase by crowding the enzyme with PEG resulted in essentially the same effect on trout muscle PFK kinetics. Both produced a marked increase in enzyme specific activity, and enzyme affinity for F6P, as well as reducing inhibition by ATP (Figs 2 and 3). Thus, the results clearly show that the regulatory behavior of trout muscle PFK is highly dependent on its concentration. It can be concluded that the allosteric regulation of fish muscle PFK is ultimately affected by its high concentration in vivo and this phenomenon, similarly to the mammalian enzyme (Bosca et al., 1985), implies a substantially higher enzyme affinity for F6P and lower sensitivity to ATP inhibition in vivo, compared with the kinetics displayed in vitro.

Fig. 4. pH profiles of trout muscle PFK. Assays were carried out in the presence of 5 mM ATP and 1 mM F6P in 20 mM imidazole buffer over the pH range 5.5-8.5. Circles show the effect of pH on enzyme activity in the absence (●) and presence (○) of 10% PEG, respectively. Triangles (▲), show enzyme activity assayed in the presence of 0.1 mM AMP. The PFK concentration was 0.6 μg/ml. Activity is plotted relative to the maximal activity of PFK in the presence of PEG at the pH optimum of 7.8.
It is well known that enzyme concentration affects the aggregation state of animal PFK. Enzyme activators have also been found to promote the aggregation of PFK into a polymer state (Reinhart and Lardy, 1980). Increases in enzyme affinity for F6P as well as for allosteric activators were found to be associated with such enzyme aggregation (Reinhart and Hartleip, 1987). The addition of PEG into the assay mixture affected enzyme kinetics (F6P saturation curve, ATP inhibition) and pH dependence in the same way as did the addition of AMP, inorganic phosphate, or other allosteric activators of the enzyme (Su and Storey, 1992). Other compounds which are believed to “crowd” proteins in dilute solution acted the same way as did PEG (Table 2). Like allosteric activators of the enzyme, PEG significantly increased the activity of trout muscle PFK, especially at low pH. It has been reported that the active tetramer of muscle PFK undergoes reversible pH-induced disassociation into an inactive dimer (Pavelich and Hammes, 1973; Hofer and Krystek, 1975) but polymerization of PFK was enhanced by enzyme activators. Therefore, PEG increases the enzyme concentration and consequently, stabilizes the enzyme by preventing it from depolymerizing which occurs at low pH. A study using fluorescence polarization of rat liver PFK covalently bound to pyrenebutyrate confirmed that PEG causes a substantial association of the enzyme (Reinhart and Hartleip, 1987). These studies, as well as the present work, suggest that changes in the state of aggregation of PFK may be considered to be a regulatory device by noncovalent interconversion.

The present results agree with prior observations by several investigators which suggested a concentration dependence of mammalian PFK activity (Reinhart, 1980; Bosca et al., 1985; Reinhart and Hartleip, 1987). Among nonmammalian PFKs, only the yeast enzyme has been previously reported to have such concentration-dependent activity. The enzyme exhibiting a significant decrease in its sensitivity to ATP inhibition when measured in situ or by using the purified enzyme at a physiological protein concentration or in the presence of PEG (Aragon and Sanchez, 1985; Bar et al., 1990). However, no effect on enzyme concentration has been detected with a nonallosteric PFK, such as that from the slime mold Dictyostelium discoideum, which lacks cooperative interactions and is not sensitive to any of the typical effectors of PFK (Baumann and Wright, 1968; Aragon et al., 1986). Therefore, regulatory properties may be essential for the enzyme to exhibit a concentration-dependent effect on enzyme kinetics.

Acknowledgements—Thanks to J. M. Storey for critical commentary on the manuscript. The research was supported by an NSERC (Canada) research grant to K.B.S.

REFERENCES

Protein concentration influences PFK kinetics.


