Pergamon

1357-2725(95)00104-2

Int. J. Biochem. Cell Biol. Vol. 27, No. 12, pp. 1277–1283, 1995
Copyright © 1995 Elsevier Science Ltd
Printed in Great Britain. All rights reserved
1357-2725/95 $9.50 + 0.00

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, $S_{0.5}$ 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

Int. J. Biochem. Cell Biol. (1995) **27**, 1277–1283

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 multimodulated 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

*Present address: Human Genome Sciences, Inc., 9410 Key West Ave, Rockville, MD 20850, U.S.A.
†Author to whom all correspondence should be addressed.
Received 23 February 1995; accepted 14 July 1995.

many intracellular enzymes are present *in vivo* 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 vivo* 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–polyacryamide 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 $MgCl_2$, 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 $MgCl_2$ 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 ($S_{0.5}$) and Hill coefficients (n_H) were determined by fitting the data to the Hill equation using a nonlinear least-squares regression computer program (Brooks, 1992). Inhibitor constants (I_{50}) were obtained from plots of reaction velocity vs inhibitor concentration. Results are presented

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

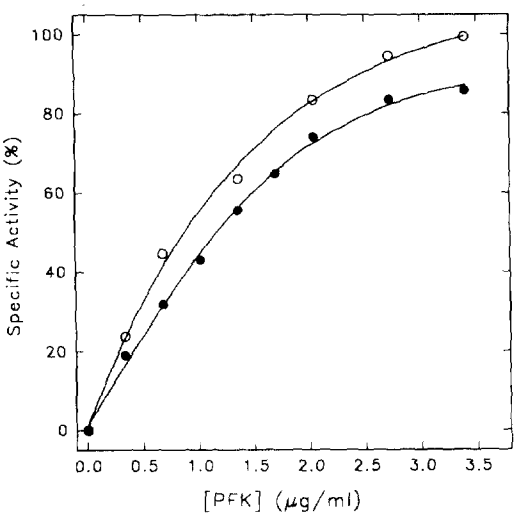

Fig. 1. Influences of PFK concentration on enzyme specific activity. Assays were carried out in the presence of 7.5 mM ATP and 0.20 mM F6P at pH 7.2 in the absence (●) vs presence (○) of 10% (w v) PEG. Specific activity is shown as a percentage of the highest activity found with 3.4 μg/ml PFK.

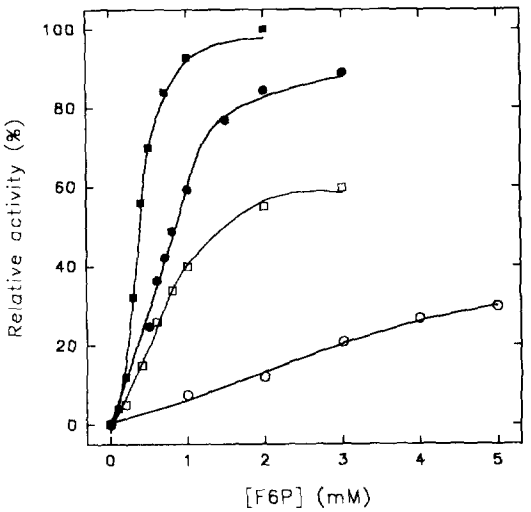

Fig. 2. Effect of enzyme and PEG concentrations on the F6P saturation of trout muscle PFK. Enzyme assays were carried out in 20 imidazole buffer, pH 7.2 in the presence of 7.5 mM ATP. PFK concentrations were 0.1 μg/ml (●, ○) and 0.6 μg/ml (■, □), in the absence (○, □) or presence of 10% (w/v) PEG (●, ■) added in the assay buffer. Results are the mean of three determinations; standard error bars are within the dimensions of the symbols used. Data are plotted relative to the maximal activity reached in the presence of 0.6 μg/ml PFK + 10% w/v PEG (■).

sigmoidal fructose-6-phosphate (F6P) saturation curve ($n_H > 1$) was found. Based on enzyme purification data (Su and Storey, 1992), the *in vivo* 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 $S_{0.5}$ 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/V_{max} 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 I_{50} value for Mg.ATP increased by nearly 5-fold when PFK concentration was increased from 0.1 to 0.6 μg/ml. I_{50} 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.

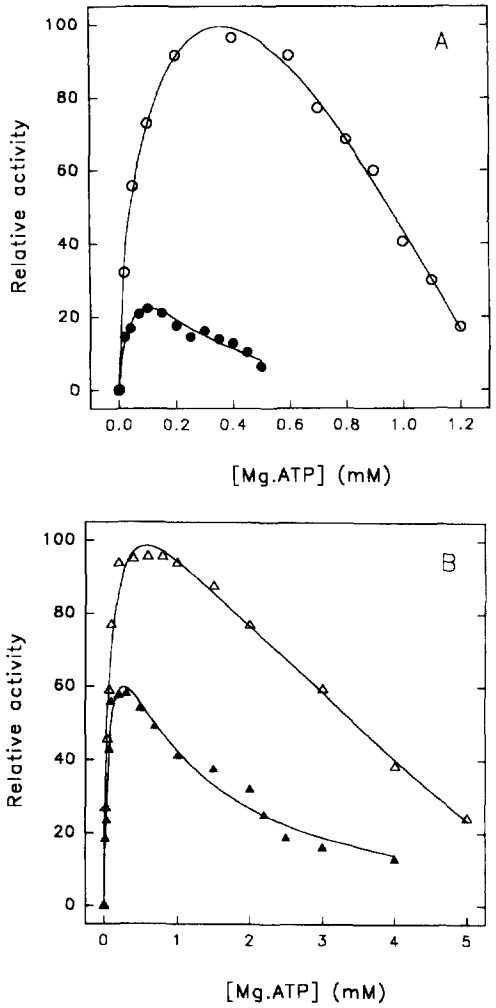

Fig. 3. Influence of enzyme and PEG concentrations on PFK inhibition by ATP. PFK was assayed in the presence of 0.20 mM F6P in imidazole buffer, pH 7.2 in the absence (A) and presence (B) of 10% (w/v) PEG. Purified PFK was used at a concentration of 0.1 μg/ml (●, ▲) or 0.6 μg/ml (○, △). Data is plotted relative to the maximal activity of PFK in the presence of 10 mM F6P + 2 mM ATP.

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 vivo*. 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

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

[PFK] (μg/ml)	[PEG] (%, w v)	S_{50} for F6P[1] (mM)	I_{50} for ATP[2] (mM)	n_H	V_{max} (relative)
0.1	0	3.54 ± 0.17	–	1.46 ± 0.07	1.00 ± 0.04
0.6	0	0.73 ± 0.02[a]	–	1.91 ± 0.04[a]	1.26 ± 0.02[a]
0.1	10	0.75 ± 0.01[b]	–	2.39 ± 0.07[b]	1.93 ± 0.03[b]
0.6	10	0.37 ± 0.02[a,b]	–	2.98 ± 0.06[a,b]	2.04 ± 0.01[a,b]
0.1	0	–	0.02 ± 0.01	1.00 ± 0.03	1.00 ± 0.03
0.6	0	–	0.94 ± 0.03[a]	4.42 ± 0.25[a]	4.35 ± 0.08[a]
0.1	10	–	1.87 ± 0.05[b]	1.73 ± 0.06[b]	2.31 ± 0.06[b]
0.6	10	–	3.23 ± 0.02[a,b]	2.12 ± 0.09[b]	4.33 ± 0.10[a]

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, $n = 3$. [a]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), $P < 0.05$; [b]significantly difference from the corresponding value without PEG.

(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*.

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

Addition	Concentration (%)	Relative Activity	v/V_{max}
None		1.00 ± 0.04	0.126
PEG	5 (w v)	2.61 ± 0.21	0.316
	10	4.74 ± 0.63	0.574
	15	8.22 ± 0.80	0.994
	20	8.26 ± 0.83	1.000
DMSO	2.5 (v v)	1.96	0.237
	5.0	3.74 ± 0.11	0.453
	10	8.54 ± 0.11	1.034
	20	9.13	1.015
Glycerol	5 (w v)	1.46 ± 0.07	0.174
	10	2.61 ± 0.09	0.315
	20	3.59 ± 0.04	0.434
	30	3.96 ± 0.09	0.482
	40	4.41 ± 0.07	0.534

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. V_{max} 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, $n = 1$.

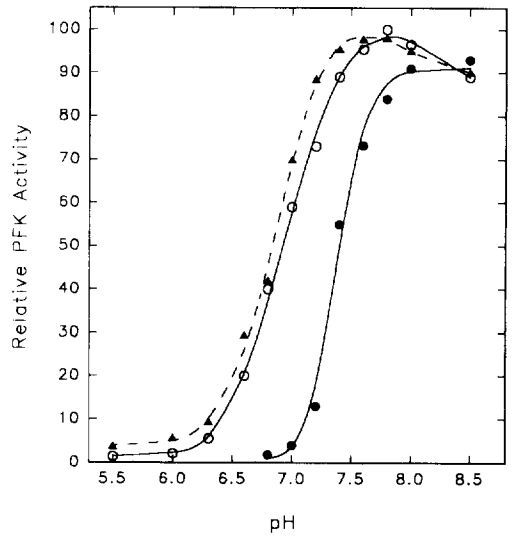

Fig. 4. pH profiles of trout muscle PFK. Assays were carried out in the presence of 5 mM ATP and 1 mM F6P in 20 mM imidazole buffer over the pH range 5.5–8.5. Circles show the effect of pH on enzyme activity in the absence (●) and presence (○) of 10% PEG, respectively. Triangles (▲), show enzyme activity 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

Aragon J. J. and Sanchez V. (1985) Enzyme concentration affects the allosteric behavior of yeast phosphofructokinase. *Biochim. Biophys. Res. Commun.* **131,** 849–855.

Aragon J. J. and Sols A. (1991) Regulation of enzyme activity in the cell: effect of enzyme concentration. *FASEB J.* **5,** 2945–2950.

Aragon J. J., Feliu J. E., Frenkel R. A. and Sols A. (1980) Permeabilization of animal cells for kinetic studies of intracellular enzymes: *in situ* behavior of the glycolytic enzyme of erythrocytes. *Proc. Nat. Acad. Sci. U.S.A.* **77,** 6324–6328.

Aragon J. J., Sanchez V. and Boto L. (1986) Fructose-2,6-bisphosphate in *Dictyostelium discoideum*, independence of cyclic AMP production and inhibition of fructose-1,6-bisphosphatase. *Eur. J. Biochem.* **161,** 757–761.

Arakawa T. and Timasheff S. N. (1985) Mechanism of poly(ethylene glycol) interaction with proteins. *Biochemistry* **24,** 6756–6771.

Bar J., Martinez-Costa O. H. and Aragon J. J. (1990) Regulation of phosphofructokinase at physiological concentration of enzyme studied by stopped-flow measurements. *Biochim. Biophys. Res. Commun.* **167,** 1214–1220.

Baumann P. and Wright B. E. (1968) The phosphofructokinase of *Dictyostelium discoideum*. *Biochemistry* **7,** 3653–3661.

Bosca L., Aragon J. J. and Sols A. (1985) Modulation of muscle phosphofructokinase at physiological concentration of enzyme. *J. Biol. Chem.* **260,** 2100–2107.

Bradford M. M. (1976) A rapid, sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254.

Brooks S. P. J. (1992) A simple computer program with statistical tests for the analysis of enzyme kinetics. *BioTechniques* **13,** 906–911.

Brooks S. P. J. and Storey K. B. (1988) Subcellular enzyme binding on glycolytic control: *in vivo* studies with fish muscle. *Am. J. Physiol.* **255,** R289–R294.

Gekko K. and Timasheff S. N. (1981) Thermodynamic and kinetic examination of protein stabilization by glycerol. *Biochemistry* **20,** 4677–4686.

Helmerhorst E. and Stokes H. H. (1980) Microcentrifuge desalting: a rapid, quantitative method for desalting small amounts of protein. *Anal. Biochem.* **104,** 130–135.

Hofer H. W. and Krystek E. (1975) Determination of the kinetic parameters of phosphofructokinase dissociation. *FEBS Lett.* **53,** 217–220.

Knoll D. and Hermans J. (1983) Polymer–protein interactions: comparison of experiment and excluded volume theory. *J. Biol. Chem.* **258,** 5710–5715.

Lee J. C. and Lee L. L. Y. (1987) Thermal stability of proteins in the presence of poly(ethylene glycol). *Biochemistry* **26,** 7813–7819.

Ling K. H., Marcus F. and Lardy H. A. (1965) Purification and some properties of rabbit skeletal muscle phosphofructokinase. *J. Biol. Chem.* **240,** 1893–1899.

Medina R., Aragon J. J. and Sols A. (1985) Effects of polyethylene glycol on the kinetic behavior of pyruvate kinases and other potentially regulatory liver enzymes. *FEBS Lett.* **180,** 77–80.

Middaugh C. R., Tisel W. A., Haire R. N. and Rosenberg A. (1979) Determination of the apparent thermodynamic activities of saturated protein solutions. *J. Biol. Chem.* **254,** 9724–9730.

Miekka S. I. and Ingham K. C. (1978) Influence of self-association of proteins on their precipitation by poly(ethylene glycol). *Arch. Biochem. Biophys.* **191,** 525–526.

Milligan C. L. and Wood C. M. (1987) Muscle and liver intracellular acid–base and metabolite status after strenuous activity in the inactive, benthic starry flounder *Platichthys stellatus*. *Physiol. Zool.* **60,** 54–68.

Mortberg M. and Neujahr H. Y. (1987) In situ and *in vitro* kinetics of phenol hydrolase. *Biochem. Biophys. Res. Commun.* **146,** 41–46.

Ovadi J., Aragon J. J. and Sols A. (1986) Phosphofructokinase and fructosebisphosphatase from muscle can interact at physiological concentration with mutual effects on their kinetic behavior. *Biochem. Biophys. Res. Commun.* **135,** 852–856.

Pavelich M. J. and Hammes G. G. (1973) Aggregation of rabbit muscle phosphofructokinase. *Biochemistry* **12,** 1408–1414.

Ramados C. S., Luby L. J. and Uyeda K. (1976) Affinity chromatography of phosphofructokinase. *Arch. Biochem. Biophys.* **175,** 487–494.

Reinhart G. D. (1980) Influence of polyethylene glycols on the kinetics of rat liver phosphofructokinase. *J. Biol. Chem.* **255,** 10576–10578.

Reinhart G. D. and Hartleip S. B. (1987) Perturbation of the quaternary structure and allosteric behavior of rat liver phosphofructokinase by polyethylene glycol. *Arch. Biochem. Biophys.* **258,** 65–76.

Reinhart G. D. and Lardy H. A. (1980) Rat liver phosphofructokinase: kinetic and physiological ramifications of the aggregation behavior. *Biochemistry* **19,** 1491–1495.

Sols A., Castano J. G., Aragon J. J., Domenech C., Lazo P. A. and Nieto A. (1981) Multimodulation of phosphofructokinase in metabolic regulation. In *Metabolic Interconversion of Enzymes* (Edited by Holzer H.), pp. 111–123. Springer Verlag, Berlin.

Sols A. and Marco R. (1979) Concentrations of metabolites and binding sites. Implications in metabolic regulation. *Curr. Top. Cell. Regul.* **2,** 227–273.

Srere P. A. (1987) Complex of sequential metabolic enzymes. *Ann. Rev. Biochem.* **56,** 89–124.

Srivastava D. K. and Bernhard S. A. (1987) Biophysical chemistry of metabolic reaction sequences in concentrated enzyme solution and in the cell. *Ann. Rev. Biophys. Biophys. Chem.* **16,** 175–204.

Storey K. B. (1987) Regulation of liver metabolism by enzyme phosphorylation during mammalian hibernation. *J. Biol. Chem.* **262,** 1670–1673.

Su J. Y. and Storey K. B. (1992) Phosphofructokinase from white muscle of the rainbow trout, *Oncorhynchus mykiss*: purification and properties. *Biochim. Biophys. Acta* **1160,** 301–308.

Su J. Y. and Storey K. B. (1994) Regulation of rainbow trout white muscle phosphofructokinase during exercise. *Int. J. Biochem.* **26,** 519–528.

Uyeda K. (1979) Phosphofructokinase. *Adv. Enzymol.* **48,** 193–244.