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REGULATION OF RAINBOW TROUT WHITE MUSCLE PHOSPHOFRUCTOKINASE DURING EXERCISE

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Abstract—1. Studies of purified 6-phosphofructo-1-kinase (PFK) from white skeletal muscle of the rainbow trout (*Oncorhynchus mykiss*) were undertaken to illuminate aspects of the regulation of glycolysis in muscle under resting versus exercising conditions.

2. Trout muscle PFK was strongly inhibited by ATP at physiological levels and was regulated by a number of allosteric effectors.

3. Allosteric activators included ammonium ion, inorganic phosphate, AMP, ADP, and fructose-2,6-bisphosphate ($F2,6P_2$); these enhanced enzyme affinity for F6P and reversed inhibition by ATP.

4. Changes in pH also played a major role in PFK regulation; as pH decreased from 7.2 to 6.6 (mimicking the pH decrease during exhaustive exercise), not only was enzyme activity reduced, but the reaction cooperativity increased as well.

5. The negative effect of reduced pH, however, was fully compensated for by the rise in the levels of positive modulators, such AMP following exercise.

6. When assayed under substrate and effector concentrations that reflected "resting" vs "exercised" situations in muscle, the $S_{0.5}$ values for F6P were reduced by 50- and 123-fold, respectively, to values of 0.068 and 0.15 mM, both close to the physiological levels of F6P.

Oncorhynchus mykiss Skeletal muscle glycolysis Exercise metabolism Fish muscle glycolysis

INTRODUCTION

The physiological and metabolic responses of fish skeletal muscle to either endurance or burst swimming have been characterized by a number of authors (Dobson *et al.*, 1987; Parkhouse *et al.*, 1988; Storey, 1991). In fish, as in all vertebrates, the highest levels of exercise performance are achieved anaerobically (Jones, 1981). At relatively low swimming speeds, fish muscle is powered primarily by red muscle fibers utilizing aerobic, oxidative pathways of ATP generation. However, burst swimming is powered by white muscle using creatine phosphate hydrolysis over the first few seconds and followed by glycogenolysis and the production of ATP via anaerobic glycolysis (Dobson *et al.*, 1987; Storey, 1991). Thus, a study of the regulation of glycolysis in muscle is important for understanding the physiological and metabolic responses of fish skeletal muscle to either endurance or burst swimming.

Phosphofructokinase (PFK, ATP: D-Fructose-6-phosphate-1-phosphotransferase, EC 2.7.1.11) is well known to be an important site of regulation of glycolysis in vertebrate muscle (Newsholme and Start, 1976; Uyeda, 1979). Detailed knowledge of the molecular mechanisms of enzyme control is therefore essential for a full understanding of the regulation of the glycolytic pathway. The regulation of PFK activity *in vivo* is very complicated since the enzyme is modulated by a variety of effectors (Uyeda, 1979; Sols *et al.*, 1981; Hers and Hue, 1983). PFK is

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Abbreviations: PFK, 6-phosphofructo-1-kinase; $F2,6P_2$, fructose-2,6-bisphosphate; K_m, Michaelis constant; K_a, activation coefficient; I_{50}, concentration of inhibitor which reduces enzyme activity by 50%.

highly responsive to the energy status of the cell through activation by AMP, ADP and inorganic phosphate and inhibition by high levels of its substrate, ATP. The enzyme is also activated by sugar bisphosphates and inhibited by citrate and PEP. PFKs purified from a number of sources are also extremely sensitive to pH change. A decrease in pH not only decreases the activity of PFK, but can lead to very important modifications of the regulatory behavior of the enzyme. It is generally believed that changes in pH shift the equilibrium between protonated and unprotonated forms of PFK (Pettigrew and Frieden, 1979a,b; Evans *et al.*, 1981). Positive effectors such as AMP bind primarily to the unprotonated form, whereas negative modulators such as ATP and citrate bind preferentially to the protonated form.

Exhaustive burst exercise in the rainbow trout results in a large accumulation of lactate and protons within the working muscle along with a depletion of glycogen, ATP and creatine phosphate stores (Dobson *et al.*, 1987; Milligan and Wood, 1986a,b; Tang and Boutilier, 1991). It is appreciated that changes in the levels of metabolites and intracellular pH in muscle during exercise affects glycolysis through the control of PFK activity. To understand the regulation of PFK, and hence the control of glycolysis, kinetic studies of the purified PFK from white skeletal muscle of rainbow trout were undertaken. The enzyme was also analyzed under metabolite and pH conditions that mimic the metabolic situation in resting versus exercised muscle. The results indicate that changes in pH and concentrations of positive modulators play a very important role in regulating PFK in the white muscle of rainbow trout during contractile activity.

MATERIALS AND METHODS

Animals and materials

Freshwater adapted rainbow trout (*Oncorhynchus mykiss*) were maintained on a 12 hr/12 hr dark/light cycle in dechlorinated Ottawa tap water (15°C) at the aquatic animal holding facilities of the Department of Biology, University of Ottawa, for at least 1 month following delivery from a commercial supplier (Linwood Acres Trout Farm, Campbellcroft, Ontario). The animals were fed once daily with commercially prepared trout food. All animals were quickly killed by a single sharp blow to the head. White muscle tissue was excised and frozen in liquid nitrogen and then kept at −80°C until used.

ATP, ADP, AMP and NADH were from Boehringer Mannheim Corp., Montreal, PQ. The remainder of chemicals, biochemicals and enzymes were from Sigma Chemical Co., St Louis, Mo.

Enzyme assays

Activity of PFK was determined using the $F1,6P_2$ coupled assay previously described (Su and Storey, 1992). The assay solution contained 20 mM imidazole-HCl, 20 mM KCl, 5 mM $MgCl_2$, 0.15 mM NADH, 0.5 U aldolase, 0.5 U triosephosphate isomerase, and 1 U glycerol-3-phosphate dehydrogenase in a total volume of 1 ml with F6P, ATP and pH as indicated in the "Results" section. All ATP and citrate stock solutions contained added $MgCl_2$ in 1:1 molar amounts. Ammonium sulfate in the auxiliary enzymes was removed by dialysis overnight against 20 mM imidazole-HCl, pH 7.2 or centrifugation through a small column of Sephadex G-25 before use (Helmerhorst and Stokes, 1980). Assays were performed at 22°C using a Gilford 240 spectrophotometer.

Kinetic parameters are defined as following: K_m (Michaelis constant), the concentration of substrate (MgATP) at which the enzyme reaction proceeded at 50% of its maximal velocity; $S_{0.5}$, the concentration of F6P at which PFK exhibits half of the V_{max}; I_{50}, the concentration of inhibitor at which the enzyme reaction is inhibited by 50%; K_a, the concentration of activator which produces 50% of its maximal activation; n_H, the Hill coefficient is the slope of Hill plot of log $(v/(V_{max}-v))$ vs log [substrate]. $S_{0.5}$ and n_H values were determined by fitting the data to the Hill equation through a nonlinear least squares regression computer kinetics program (Brooks, 1992). A modified Hill equation that introduced a v_0 term (rate at zero activator concentration) was used for determining K_a values. I_{50} values were obtained from plots of rate vs [inhibitor]. Results are presented as means ± SEM with $n = 3$ (at least) determinations on separate preparations of the purified enzyme. Tests for significant differences between values used the Student's t-test.

Enzyme preparation

Trout white muscle PFK was purified as previously described (Su and Storey, 1992) to a specific activity of 70 U/mg or greater. The

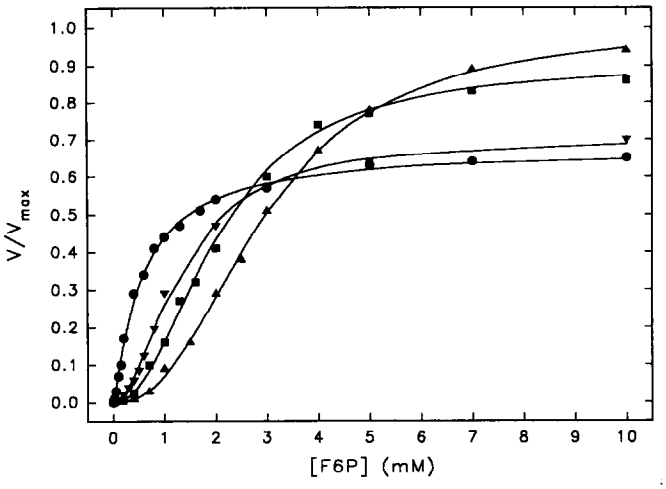

Fig. 1. F6P saturation curves for trout muscle PFK at different ATP concentrations. PFK was assayed at pH 7.2 with ATP concentrations: 0.5 mM (●), 1.0 mM (▼), 3.5 mM (■), and 7.5 mM (▲). The V_{max} value was taken under the condition of 7.5 mM ATP and infinite concentration of F6P which was derived from non-linear least square regression through a computer program.

purified enzyme was homogeneous as judged by SDS–polyacrylamide gel electrophoresis. For kinetic experiments, PFK concentration was about 0.6 μg/ml in the assay cuvette.

RESULTS

Figure 1 shows the effect of F6P concentration on the activity of trout white muscle PFK at pH 7.2. Similar F6P saturation curves were also observed at pH 6.6. The kinetic parameters for F6P are summarized in Table 1. It can be seen that the kinetic plots for the enzyme are sigmoidal when the activity is represented as a function of F6P concentration at physiological intracellular pH values for both "resting" (pH 7.2) and "exercised" (pH 6.6) situations. ATP, the other substrate of the reaction, enhanced the sigmoidal appearance of the curves when its concentration was increased. The cooperative effects were characterized by Hill coefficients (n_H) which were all greater than unity at ATP concentrations ranging from 0.5 to 7.5 mM. At pH 7.2, there was a progressive increase (from 1.14 to 3.11) in the Hill coefficient as ATP concentration rose; a similar trend was also observed at pH 6.6 (Table 1). Increasing concentrations of ATP also increased the $S_{0.5}$ values for F6P, decreasing enzyme affinity for F6P at both high and low pH values. When ATP levels were increased from 1.0 to 7.5 mM, the $S_{0.5}$ values increased by approx. 3-fold at both high and low pH values (Table 1).

Table 1. F6P kinetic parameters of trout muscle PFK at different pH values with varying concentrations of ATP

pH	ATP (mM)	$S_{0.5}$ F6P (mM)	n_H	n
7.2	0.5	0.57 ± 0.03	1.14 ± 0.05	(3)
	1.0	1.36 ± 0.01	1.86 ± 0.09	(4)
	3.5	2.17 ± 0.12	2.14 ± 0.08	(3)
	7.5	3.16 ± 0.11	3.11 ± 0.45	(5)
6.6	1.0	8.86 ± 0.52[a]	4.06 ± 0.30[b]	(4)
	3.5	18.9 ± 0.48[a]	5.02 ± 0.52[b]	(4)
	7.5	26.3 ± 0.30[a]	4.62 ± 0.39[b]	(3)

All $S_{0.5}$ and Hill coefficient (n_H) values are expressed as means ± SEM with n values as shown.

[a]Significantly different from the corresponding values at pH 7.2, $P < 0.001$ by Student's t-test; [b]$P < 0.05$.

The change in assay pH from 7.2 to 6.6 had a strong effect on the substrate affinity and reaction cooperativity of the enzyme. From Table 1, it is clear that trout muscle PFK exhibited higher substrate affinity (lower $S_{0.5}$ F6P) and lower reaction cooperativity (lower n_H values) at the physiological pH for resting trout muscle (pH = 7.2) than at the pH value found in muscle after exhaustive exercise (pH = 6.6).

The effect of ATP on enzyme activity is better observed in Fig. 2. The enzyme showed a K_m for ATP of 30 ± 4 μM ($n = 6$) with the Hill coefficient not significantly different from unity. The K_m values for ATP were not influenced by the concentration of F6P. However, the maximal reaction velocity and the ATP inhibition constant (I_{50}) were affected substantially by changing F6P concentration. At higher concentrations ATP inhibits PFK and Fig. 2 shows

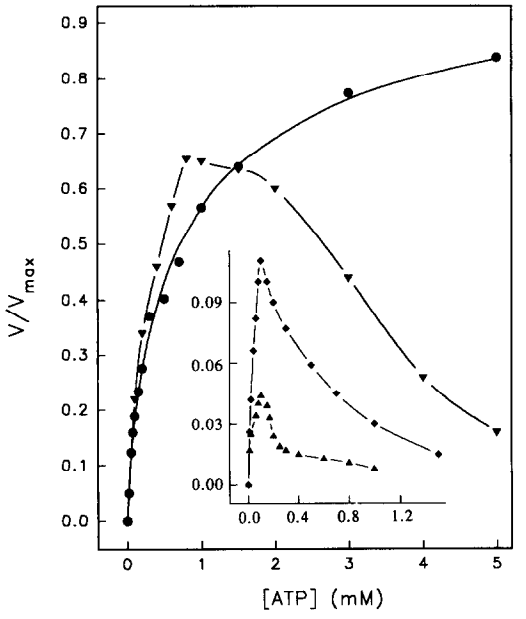

Fig. 2. Effect of ATP concentration on the activity of PFK from trout muscle. PFK activity was assayed in 20 mM imidazole–HCl buffer, pH 7.2, at 22°C in the absence of PFK effectors and with different concentrations of F6P: 10 mM (●), 1.0 mM (▼), 0.20 mM (◆, inset), and 0.075 mM (▲, inset). The V_{max} value was taken as the maximal reaction velocity when 10 mM F6P was employed.

that this is also dependent on F6P concentrations. The apparent I_{50} for ATP ranged from 0.25 to 3.57 mM in the presence of 0.075 mM (physiological concentration in resting muscle, Dobson *et al.*, 1987) to 1.00 mM F6P. When F6P concentration was elevated to 5.0 mM or above, no ATP inhibition was observed over the physiological concentration range of ATP up to 10 mM (Table 2). Along with the increase in I_{50} values, ATP inhibition also showed a decreasing kinetic cooperativity with increasing F6P concentration. When pH was decreased, ATP inhibition was much stronger. At pH 6.6, an I_{50} value for ATP of 0.23 ± 0.02 mM ($n = 3$) was found in the presence of 1.0 mM F6P.

Table 2. Apparent I_{50} values for ATP at different levels of F6P

F6P (mM)	Relative V_{max}	I_{50} (mM)	n_H
0.075	1.00	0.25 ± 0.01	5.65 ± 0.29
0.16	2.70	0.57 ± 0.02	1.83 ± 0.09
0.40	8.10	0.99 ± 0.09	1.21 ± 0.16
1.00	15.8	3.57 ± 0.05	4.39 ± 0.29
5.00	25.8	NI	
10.0	26.5	NI	

All values are expressed as the average of two separate determinations. The concentration of PFK used was 0.6 μg/ml in the assay mixture. NI—no inhibition at ATP concentrations as high as 10 mM.

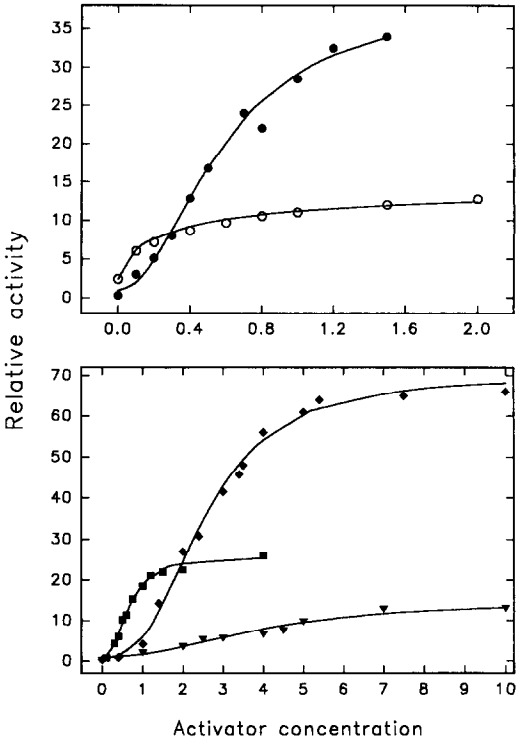

Fig. 3. Effects of modulators on trout muscle PFK activity. Enzyme assays were run at pH 7.2 in imidazole buffer under inhibitory or non-inhibitory concentrations of ATP. Upper part of the graph shows the effect of AMP on the activity of muscle PFK. (●) Deinhibition by AMP when the enzyme was assayed under high (1.0 mM) ATP; (○) the activation effect of AMP at low non-inhibitory ATP (0.06 mM ATP). Lower part shows deinhibition of muscle PFK by ammonium chloride (▼), inorganic phosphate (◆), and F2,6P_2 (■) at high ATP (1.0 mM). All assays contained 0.20 mM F6P. Activator concentrations are in mM except for F2,6P_2 which is in μM. Data are means of duplicate determinations with error bars contained within the symbols. Enzyme activity in the absence of activators was set at 1.

In addition to the effects of F6P and ATP on PFK, the influences of several metabolites on the trout muscle enzyme activity were evaluated. Ammonium ion, inorganic phosphate, AMP, ADP, KCl and F2,6P_2 were all found to stimulate the enzyme. The stimulatory effects of these metabolites were highly dependent on the concentration of ATP. Figure 3 shows the influence of some effectors on trout muscle PFK activity; the action of AMP is shown under high and low levels of ATP. The kinetic parameters of the enzyme modulators are summarized in Table 3. Velocity vs activator concentration relationships were all strongly sigmoidal. F2,6P_2 was the most effective activator with its activation constant less than 1 μM in assays containing inhibitory levels of ATP. When ATP concentration was decreased to the non-inhibitory range, only

Table 3. Apparent K_a values for positive modulators of trout muscle PFK

	Activator	K_a	n_H	Fold activation
(A)	NH_4Cl	4.03 ± 0.14	2.06 ± 0.06	16 ± 1
	AMP	0.60 ± 0.01	2.02 ± 0.01	38 ± 2
	ADP	0.84 ± 0.05	1.76 ± 0.22	13 ± 1
	P_i	2.54 ± 0.10	2.70 ± 0.10	71 ± 2
	$F2,6P_2$	0.66 ± 0.02	2.24 ± 0.05	27 ± 1
	KCl*	81.8	ND	6
(B)	NH_4Cl	0.42 ± 0.05	1.08 ± 0.21	16 ± 1
	AMP	0.34 ± 0.03	0.82 ± 0.10	6.5 ± 0.2
	P_i	NA		
	ADP	NA		
	$F2,6P_2$	NA		

Activation constants are expressed as mean ± SEM based on at least three determinations except for KCl (*) which is a single determination. The values of K_a are in mM except for that of $F2,6P_2$ which is in μM. NA—no activation effect was observed; ND—not determined. (A) PFK was assayed under an inhibitory concentration of ATP, 3.4 mM with 0.16 mM F6P; (B) PFK assays were run under a non-inhibitory concentration of ATP, 0.06 mM with 0.20 mM F6P.

ammonium ion and AMP retained their activating effects. Comparing inhibitory (3.4 mM) vs non-inhibitory (0.06 mM) ATP concentrations, the K_a value for ammonium chloride decreased by 9-fold from 4.03 to 0.42 mM, whereas that for AMP decreased by 2-fold. The fold activation of PFK by AMP was also much reduced at low ATP levels. The deinhibition effects of ammonium, phosphate, AMP, ADP, and $F2,6P_2$ were also noticed at low assay pH (6.6) where their K_a values were 1.45 mM, 3.31 mM, 0.42 mM 0.18 mM, and 0.12 μM, respectively, in the presence of 1.0 mM F6P and 0.5 mM ATP.

Table 4. Citrate and PEP inhibition of trout muscle PFK

Substrate concentration (mM)	I_{50} values of inhibitors	
	PEP	citrate
1.0 ATP + 0.2 F6P	0.75 ± 0.14	0.027 ± 0.003
0.06 ATP + 0.2 F6P	0.59 ± 0.11	0.113 ± 0.009[a]

Values are in mM and are expressed as means ± SEM, $n = 3$. PFK was assayed at pH 7.2 under low, non-inhibitory ATP or high, inhibitory ATP concentrations.
[a]Significantly different from the corresponding value under high ATP concentration, $P < 0.01$.

Table 4 shows the effects of PEP and citrate, two well known inhibitors of PFK, on the kinetics of the enzyme. Citrate was a much more potent inhibitor of trout muscle PFK at high ATP concentration than at low levels of ATP. At a F6P concentration of 0.2 mM, the apparent I_{50} of citrate decreased by 4-fold in the presence of 1.0 mM (inhibitory) vs 0.06 mM (non-inhibitory) concentrations of ATP. However, the I_{50} of PEP did not change significantly with a change in ATP concentration. The inhibitory effects of citrate and PEP were reversed by the addition of allosteric activators of the enzyme (data not shown).

Figure 4 shows the effect of positive or negative enzyme modulators on the ATP inhibition of trout muscle PFK. The changes in ATP I_{50} values are summarized in Table 5. Positive effectors decreased the affinity of ATP for the allosteric binding site (increased I_{50} values) whereas inhibitors increased ATP inhibition

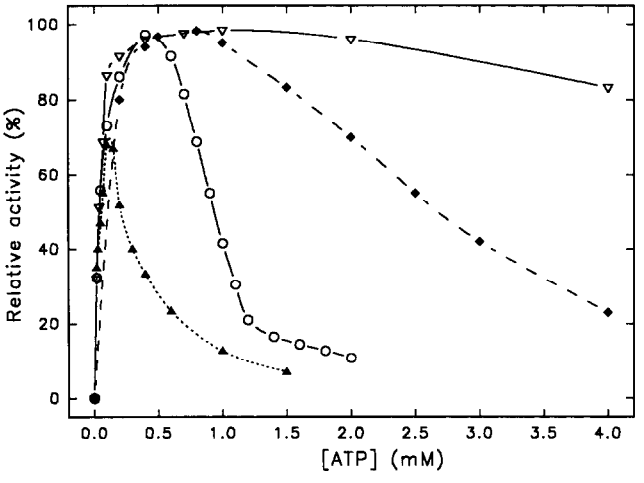

Fig. 4. The influence of modulators on the ATP saturation curves of trout muscle PFK. Enzyme assays were run at pH 7.2 with the *in vivo* concentration of F6P (0.2 mM) for resting fish muscle. (○) The PFK reaction profile in the absence of modulators. (◆) plus 0.2 μM $F2,6P_2$; (▽) plus 5 mM phosphate; (▲) plus 0.1 mM citrate.

Table 5. Effect of modulators on the apparent I_{50} values for ATP of trout muscle PFK

Effector	Concn	I_{50} (mM)	n_H	n
None	—	0.94 ± 0.03	4.42 ± 0.25	(9)
P_i	5.0 mM	9.44 ± 0.29^a	1.75 ± 0.07^a	(3)
$F2,6P_2$	0.2 μM	2.71 ± 0.03^a	2.98 ± 0.02^a	(5)
	10 μM	19.1 ± 1.4^a	1.57 ± 0.03^a	(3)
PEP	0.05 mM	1.00 ± 0.1	2.26 ± 0.78	(2)
Citrate	0.10 mM	0.39 ± 0.03^a	2.41	(2)
	0.30 mM	0.31 ± 0.03^a	2.22	(2)
	0.50 mM	0.27 ± 0.03^a	2.86	(2)

PFK was assayed in imidazole buffer, pH 7. 2 with 0.2 mM F6P. No noticeable difference was observed in the K_m values for ATP with or without either positive or negative modulators in the assay mixture.

[a]Significantly different from the control, $P < 0.0005$.

(decreased I_{50} values). For example, in the presence of 5 mM phosphate, the I_{50} value for ATP increased 10-fold from 0.94 to 9.44 mM, whereas 10 μM $F2,6P_2$ elevated the I_{50} value for ATP by 20 times to 19.1 mM. The addition of 0.5 mM citrate, however, decreased the I_{50} of ATP from 0.94 to 0.27 mM. PEP at its physiological concentration did not change the ATP inhibition significantly. Activators also reduced the Hill coefficient of the ATP inhibition curves. However, with or without allosteric activators or inhibitors, the K_m for ATP showed no significant change from a mean value of 30 ± 4 μM.

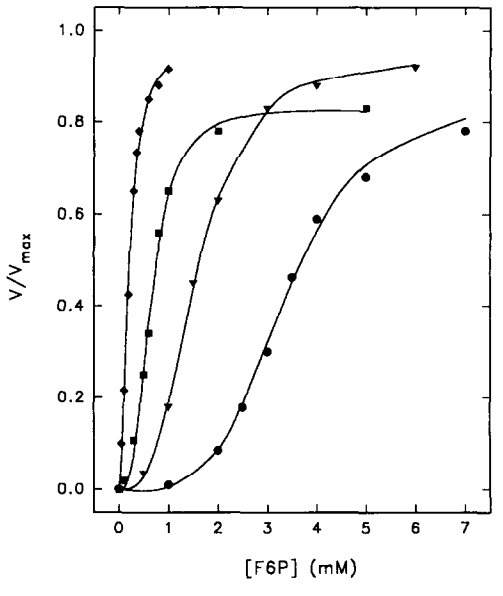

Fig. 5. Effect of activators on the substrate (F6P) affinity of trout muscle PFK. PFK was assayed in 20 mM imidazole–HCl buffer, pH 7.2, in the presence of 7.5 mM ATP which is the physiological concentration for resting trout. (●) The effect of the concentration of F6P on the PFK activity in the absence of any activator (control); (▼) plus 0.5 mM IMP; (■) plus 1.0 mM ADP; (◆) plus 10 μM of $F2,6P_2$.

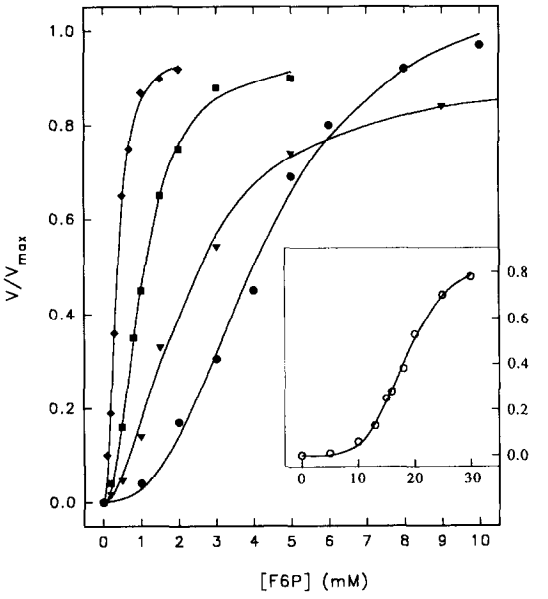

Fig. 6. Effect of activators on the enzyme activity under "exercised" conditions. Enzyme assays were run in 20 mM imidazole buffer, pH 6.6, with the concentration of ATP of 3.5 mM (the physiological level in exercised trout muscle). The V_{max} was defined as in Fig. 5. (○, inset) represent the PFK reaction profile with changing F6P concentration (control); (●) plus 5.0 mM NH_4Cl; (▼) plus 0.5 mM AMP; (■) plus 10 μM $F2,6P_2$; (◆) plus 0.5 mM AMP and 5.0 mM potassium phosphate.

The presence of allosteric activators also changed the F6P reaction curve as shown in Fig. 5. At the physiological pH and ATP concentrations in resting muscle, F6P affinity is extremely low such that at *in vivo* F6P levels the enzyme would be virtually inactive in resting trout white muscle. The same is true for the enzyme under assay conditions that mimic burst exercise (Fig. 6). However, with the addition of allosteric activators that normally increase in concentration during muscle contraction, PFK activity was increased dramatically especially at low, physiological levels of F6P. Activators decreased the $S_{0.5}$ values for F6P substantially. It is known that during muscle exercise, intracellular pH, levels of PFK substrates (F6P, ATP) and effectors are all altered. To mimic the *in vivo* regulation of PFK in trout white muscle during exercise, physiological concentrations of substrates, effectors and pH values were selected. Under physiological ATP concentrations and at pH 7.2, the addition of physiological levels of AMP, IMP (inosine monophosphate), ADP, P_i, NH_4^+, and $F2,6P_2$ decreased $S_{0.5}$ for F6P by 50-fold from 3.39 to 0.068 mM (Table 6). The comparable effect at pH 6.6 was a 123-fold decrease in the $S_{0.5}$ F6P (from 18.5 to

Table 6. Effect of modulators on the $S_{0.5}$ for F6P of trout muscle PFK at pH 7.2 under "resting" conditions

Modulators	$S_{0.5}$	n_H
None (control)	3.39	4.08
0.05 AMP	1.29	3.49
1.0 ADP	0.65	2.95
1.0 NH_4Cl	0.93	4.00
0.5 IMP	1.60	2.80
10 $F2,6P_2$	0.21	1.83
25 P_i	0.19	0.84
0.05 AMP, 0.5 IMP	1.41	2.71
0.05 AMP, 0.5 IMP, 1.0 ADP	0.71	2.68
0.05 AMP, 0.5 IMP, 1.0 ADP, 25 P_i, 1.0 NH_4Cl	0.078	1.45
0.05 AMP, 0.5 IMP, 1.0 ADP, 25 P_i, 1.0 NH_4Cl, 10 $F2,6P_2$	0.068	1.37

The concentrations of modulators and the $S_{0.5}$ values for F6P are in mM, except for $F2,6P_2$ which is in μM. PFK was assayed at 7.5 mM ATP, the physiological concentration for resting fish muscle. Concentrations of modulators and the pH value were mainly chosen to reflect *in vivo* values in "resting" white skeletal muscle (Dobson *et al.*, 1987; Parkhouse *et al.*, 1988; Walsh and Milligan, 1989). Results are $n = 1$.

0.15 mM) in the presence of *in vivo* levels of enzyme activators (Table 7). Activators were also found to change the F6P saturation curve from strongly sigmoidal ($n_H > 4$) to hyperbolic ($n_H = 1$) (Tables 6, 7; Figs 5, 6). The effects of activators, except for IMP, were additive in increasing substrate affinity and reaction velocity or counteracting ATP inhibition. At both pH 7.2 and 6.6, IMP weakened the activation effects of AMP and ADP implying that there were competitive interactions between IMP and the adenylates. The $S_{0.5}$ values for F6P obtained under *in vivo* "resting" and "exercised" conditions were comparable to the physiological levels of F6P in resting and exercised trout muscles, respectively.

Table 7. Effect of modulators on the $S_{0.5}$ for F6P of trout muscle PFK at pH 6.6 under "exercised" conditions

Modulators	$S_{0.5}$	n_H
None (control)	18.5	4.80
0.50 AMP	2.24	1.78
1.00 ADP	4.08	4.40
50 P_i	0.47	1.79
5.0 NH_4Cl	4.33	2.53
10 $F2,6P_2$	1.03	2.04
5.0 IMP	27.6	3.28
0.50 AMP, 5.0 P_i	0.47	1.15
0.25 AMP, 5.0 NH_4Cl	1.22	2.24
5.0 IMP, 5.0 NH_4Cl	6.24	2.52
0.25 AMP, 5.0 IMP, 5.0 NH_4Cl	1.61	4.81
0.25 AMP, 5.0 IMP, 5.0 NH_4Cl, 1.00 ADP, 50 P_i	0.19	0.61
0.25 AMP, 5.0 IMP, 5.0 NH_4Cl, 1.00 ADP, 50 P_i, 10 $F2,6P_2$	0.15	0.70

The $S_{0.5}$ values for F6P and the concentrations of modulators are in mM except for $F2,6P_2$ which is in μM. PFK was assayed under pH, ATP (3.5 mM) and modulator concentrations that were chosen to reflect *in vivo* values in "exercised" white skeletal muscle (Dobson *et al.*, 1987; Parkhouse *et al.*, 1988; Walsh and Milligan, 1989). Results are $n = 1$.

DISCUSSION

PFK purified from the white muscle of rainbow trout shows many properties that are similar to mammalian skeletal muscle PFK. The enzyme showed sigmoidal saturation kinetics with respect to F6P over the physiological pH range, as is common for native PFK of most origins (Sols *et al.*, 1981). The F6P saturation curves were right-shifted (increased $S_{0.5}$) with intensified sigmoidicity by increasing concentrations of ATP. Changes in pH and the addition of activators influenced F6P binding with the enzyme. Activators shifted the F6P saturation plots leftwards with reduced curve sigmoidicity (Figs 5, 6) whereas increased H^+ increased $S_{0.5}$ values for F6P and the n_H (Table 1). It is now generally accepted that the F6P binding site of the enzyme lies between two dimers. On the transition between the R (active) and the T (inactive) state of the enzyme, which may occur during the binding of allosteric effectors, the rigid dimers perform a contrary rotation around their common dyad axis, and this rotation couples the changes in the binding sites of F6P, hence altering F6P affinity and its kinetic cooperativity (Evans *et al.*, 1981; Schirmer and Evans, 1990).

The binding of ATP to the active site of PFK shows a hyperbolic dependence with half-saturation values in the range 10–100 μM for the enzyme from most sources (Fordyce *et al.*, 1982; Storey 1985; Buckwitz *et al.* 1990). The same holds true for the enzyme from white muscle of rainbow trout. The K_m value of ATP (30 μM) was not influenced by the level of F6P co-substrate, or the presence of allosteric activators or inhibitors of the enzyme. It is believed that the ATP-binding site is formed almost entirely by residues of the large domain of the protein, and the structure of the ATP site remains unchanged on the allosteric transition (Schirmer and Evans, 1990).

PFK from most sources is inhibited by ATP at concentrations within the physiological range (Uyeda, 1979). The enzyme isolated from white muscle of rainbow trout was strongly inhibited

by the ATP levels that are characteristic of either resting (7.5 mM) or exercised (3.5 mM) fish muscle (Dobson *et al.*, 1987). ATP inhibition was relieved by increasing levels of F6P or adding activators. Inhibition of the enzyme is assumed to be a result of ATP binding to the inactive T state and not the active R state at high levels of ATP (low affinity binding site) (Goldhammer and Paradies, 1979). However, F6P and activators preferentially bind to the active R form of the enzyme, therefore preventing ATP from binding to the inhibitory sites.

Citrate, an intermediate of the Krebs cycle, was found to be an allosteric inhibitor of trout muscle PFK, as for the enzyme from many eukaryotic organisms (Lobes and Penney, 1974; Newsholme *et al.*, 1977; Sols *et al.*, 1981). Citrate acts synergistically with ATP (Table 4). The synergism apparently results from the cooperative binding of these compounds at two distinct binding sites as reported for mammalian muscle PFK (Colombo *et al.*, 1975). The binding of ATP enhances the affinity of the enzyme for citrate and vice versa (Saier, 1987). The decrease in the enzyme activity upon the addition of citrate may also be due to a citrate-induced depolymerization of the protein (Pavelich and Hammes, 1973). Feedback inhibition by citrate appears to provide the means of coordinating glycolytic flux with TCA cycle activity. PEP was also an inhibitor of trout muscle PFK as has been found for PFK from some invertebrate muscles and plants (Uyeda, 1979; Whitwam and Storey, 1991). However, PEP inhibited the enzyme only at relatively high levels (Table 4), and since the physiological concentrations of PEP are quite low in exercising trout muscle (Dobson *et al.*, 1987), PEP probably has little effect on PFK regulation in the working muscle.

AMP and ADP strongly activated trout muscle PFK at high concentrations of ATP. However, the effect of ADP in counteracting ATP inhibition was less pronounced than that of AMP. AMP also increased enzyme activity when the level of ATP was non-inhibitory. The levels of both ADP and AMP increase significantly during exercise in trout. However, AMP is also deaminated to form IMP and NH_4^+ so that most of the decrease in ATP during exercise is reflected by an IMP increase. IMP also had an activating effect on trout muscle PFK at pH 7.2 (Fig. 5) although the effect was much weaker than that of AMP (Table 6). At pH 6.6, the condition of exercised muscle where levels of IMP would be highest, IMP had no activating effect on PFK and appeared to partly reverse the effect of other activators (Table 7). The competitive interactions between ADP, AMP, and IMP may come from a common allosteric binding site for these nucleotides as proposed for the enzyme from other sources (Evans *et al.*, 1981; Poorman *et al.*, 1984).

Inorganic phosphate, which increases 2-fold during exercise due to the hydrolysis of creatine phosphate (Moyes *et al.*, 1992), showed the greatest fold activation of PFK among the activators tested (Table 3). The physiological level of inorganic phosphate is much higher than the K_a values determined under conditions close to those *in vivo* and thus, P_i may be a very significant modulator of muscle PFK *in vivo*. Ammonium ion, whose concentration also increases substantially (5-fold) during exercise due to the deamination of AMP (Pettigrew and Frieden, 1979b; Parkhouse *et al.*, 1987), also stimulated white muscle PFK at high and low concentrations. The strong activating effects of NH_4^+ can maintain the impact of AMP activation which would otherwise be lost due to the poor activating effects of IMP.

The kinetic studies of trout muscle PFK were carried out at two pH values at 22°C: pH 7.2, reflecting the pH of resting trout muscle *in vivo*, and pH 6.6, reflecting the pH of muscle following burst-type exercise (Milligan and Wood, 1986a,b). Changes in pH were found to play an important role in the regulation of PFK activity. Lowering pH decreased the affinity of PFK for F6P (Table 1) and increased the inhibitory effect of ATP. Therefore, the well-known decrease in intracellular pH in fish muscle during exercise (Milligan and Farrell, 1986; Milligan and Wood, 1986a,b; Parkhouse *et al.*, 1987) should lead to a significant decrease of PFK activity. Instead, however, the glycolytic contribution to white muscle energy production is increased under high work rates (Driedzic and Hochachka, 1976; Parkhouse *et al.*, 1987). However, it has been repeatedly reported that known measured changes in the levels of effectors of PFK in muscle are not sufficient to bring about the many fold increase in flux through the PFK locus that occurs in working muscle (Helmreich and Cori, 1965; Dawson *et al.*, 1980). This is further compounded by the decrease in pH during muscle work and a number of authors have considered the paradox of how high rates of glycolysis can be maintained during muscle work despite a sharp drop in intracellular pH

into a range that is strongly inhibitory for PFK, the rate-limiting enzyme of the pathway (summarized in Dobson *et al.*, 1986). However, the negative effects of low pH may be compensated for by the effects of enzyme activators in the exercising muscle. Thus, the addition of activators shifts the optimal pH for trout muscle PFK to lower values (Su and Storey, 1992) and reverses the low pH-induced decrease in F6P affinity and increase in ATP inhibition (Fig. 5 vs 6).

$F2,6P_2$ is probably the most potent PFK activator (van Schaftingen, 1987) and is particularly effective in relieving the adverse effects of low pH on the enzyme (Table 5; Figs 5, 6) Although the levels of $F2,6P_2$ (about 1 μM) in trout white muscle do not change during contractile activity (Storey, 1991), the consistent presence of the activator may be a key factor in preventing or limiting inhibition of PFK as intracellular pH falls during muscle exercise. The influence of $F2,6P_2$ on PFK activity may come from a conformational change of the enzyme or/and the stabilizing effect of $F2,6P_2$ on the native enzyme.

To experimentally test whether the action of activators could sustain PFK function under the metabolic conditions of working muscle, we analyzed the individual and combined influences of various metabolites, each presented in near-physiological levels, on the $S_{0.5}$ for F6P at the pH values of resting (pH 7.2) versus exercised (pH 6.6) trout muscle (Tables 6, 7). Exercise-induced acidosis appeared to play a major role in the regulation of trout muscle PFK. Although the levels of ATP drop by more than half during exercise, the reduction in pH would lower the enzyme activity substantially as a result of the lower binding capacity of the enzyme for F6P; thus, the control $S_{0.5}$ was 5.5-fold higher at pH 6.6 (with 3.5 mM ATP) than at pH 7.2 (with 7.5 mM ATP). However, physiological levels of individual activators reduced both $S_{0.5}$ and n_H at both pH values and in combination, the accumulated effects of multiple activators largely eliminated the low pH effect on $S_{0.5}$ F6P. Thus, the F6P affinity constant in the presence of six activators was 0.07 mM at pH 7.2 and only slightly higher at 0.15 mM at pH 6.6 (Tables 6 and 7). Both of these values are very close to the physiological concentrations of F6P.

In conclusion, the fall in muscle pH during exercise may be compensated for by increases in levels of positive modulators of PFK to support the glycolytic flux rate demanded by working muscle. The positive modulators exert their effects by influencing the binding of substrates, enhancing the maximum activity and releasing the inhibitory effect of negative effectors. The most important effect of positive modulators is to reduce the $S_{0.5}$ for F6P into the range of physiological F6P concentrations and shift the F6P saturation curve from sigmoidal to hyperbolic.

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