J. Biochem. **144,** 235–244 (2008)
doi:10.1093/jb/mvn066

Purification and Kinetic Characterization of 6-Phosphofructo-1-kinase from the Liver of Gilthead Sea Bream (*Sparus Aurata*)

Dominica Mediavilla, Isidoro Metón and Isabel V. Baanante*

Departament de Bioquímica i Biologia Molecular, Facultat de Farmàcia, Universitat de Barcelona, Diagonal 643, 08028 Barcelona, Spain

Received March 5, 2008; accepted April 27, 2008; published online May 15, 2008

6-Phosphofructo-1-kinase (PFK) was purified to homogeneity from liver of gilthead sea bream (*Sparus aurata*) and kinetic properties of the enzyme were determined. The native enzyme had an apparent molecular mass of 510 kDa and was composed of 86 kDa subunits, suggesting homohexameric structure. At pH 7, *S. aurata* liver PFK (PFKL) showed sigmoidal kinetics for fructose-6-phosphate (fru-6-P) and hyperbolic kinetics for ATP. Fructose-2,6-bisphosphate (fru-2,6-P_2) converted saturation curves for fru-6-P to hyperbolic and activated PFKL synergistically with AMP. Fru-2,6-P_2 counteracted the inhibition caused by ATP, ADP and citrate. Compared to the *S. aurata* muscle isozyme, PFKL had lower affinity for fru-6-P, higher cooperativity, hyperbolic kinetics in relation to ATP, increased susceptibility to inhibition by ATP, and was less affected by AMP, ADP and inhibition by 3-phosphoglycerate, phosphoenolpyruvate, 6-phosphogluconate or phosphocreatine. The effect of starvation-refeeding on PFKL expression was studied at the levels of enzyme activity and protein content in the liver of *S. aurata*. Our findings indicate that short-term recovery of PFKL activity after refeeding previously starved fish, may result from allosteric regulation by fru-2,6-P_2, whereas combination of activation by fru-2,6-P_2 and increase in protein content may determine the long-term recovery of the enzyme activity.

Key words: glycolysis, kinetics, liver, 6-phosphofructo-1-kinase, *Sparus aurata*.

Abbreviations: fru-2,6-P_2 fructose-2,6-bisphosphate; fru-6-P, fructose-6-phosphate; PFK, 6-phosphofructo-1-kinase; PFKL, liver 6-phosphofructo-1-kinase; PFKM, muscle 6-phosphofructo-1-kinase; PMSF, phenylmethylsulphonyl fluoride.

6-Phosphofructo-1-kinase (PFK, EC 2.7.1.11) catalyses a key regulatory step in glycolysis through formation of fructose-1,6-biphosphate (fru-1,6-P_2) from fructose-6-phosphate (fru-6-P) and MgATP. Nutritional and hormonal status regulates the expression of liver PFK (PFKL), and allosteric control of PFK activity allows glycolytic flux to be responsive to the energy demands of the cell (*1–5*). In addition to catalytic sites for fru-6-P and ATP, mammalian PFK has four allosteric binding sites: activator sites for fructose-2,6-bisphosphate (fru-2,6-P_2) and AMP or ADP, and inhibitor sites for ATP and citrate (*6*). In the mammalian liver, PFK is a tetrameric enzyme with 80–85 kDa subunits that exhibits positive cooperativity for fru-6-P (*7*). In vertebrates, three loci encoding L, M and P-type PFK subunits have evolved from prokaryotic PFKs by gene duplication, tandem fusion and divergence of catalytic and allosteric sites (*8*). ATP and citrate inhibit the enzyme through interaction with sites that have evolved from a duplicated allosteric site of ancestral PFK, whereas sites for activators, fru-2,6-P_2 and AMP or ADP, have evolved from the catalytic site of the ancestral precursor (*9*). Depending on the relative expression of each subunit, specific isoforms are present in different tissues. The L subunit is the major form in liver, the M subunit is the only form present in adult skeletal muscle and the P subunit is found at high levels in brain. The three types of PFK subunits combine to form homo and heterotetramers with distinct allosteric and catalytic properties (*10*). Although the tetrameric form is the smallest catalytically active species, higher molecular weight forms have been also detected (*11*). PFK can undergo phosphorylation by cAMP-dependent protein kinase (*12*). However, phosphorylation does not seem to play an important role in the regulation of PFK activity in rat hepatocytes (*13*).

Carnivorous fish have little capacity to metabolize carbohydrates and maintain glycaemia. In these animals hyperglycaemia lasts longer than in mammals, both after glucose load and through carbohydrate feeding. This metabolic profile mimics type 2 diabetes in humans (*14*, *15*). We showed previously that the carnivorous fish *Sparus aurata* adapts the hepatic activity of PFKL and other key enzymes in glycolysis–gluconeogenesis to tolerate partial replacement of protein by carbohydrate in the diets supplied to fish in culture (*16–19*). Specifically, refeeding *S. aurata* on high carbohydrate diets raised the hepatic levels of fru-2,6-P_2, a potent allosteric activator of PFK and inhibitor of fructose-1,

*To whom correspondence should be addressed. Tel: +34-934-02-4521, Fax: +34-934-02-4520, E-mail: baanantevazquez@ub.edu

6-bisphosphatase, from barely detectable levels during starvation to the highest values ever reported (*17*, *20*). Taken together, these data indicate that regulation of carbohydrate metabolism in carnivorous fish differs from mammals. Since PFK is a key enzyme in glycolysis and has a major role in the liver to control glucose homeostasis, we addressed purification and kinetic characterization of PFK from the liver (PFKL) of the carnivorous fish *S. aurata*, which is nowadays the most extensively cultured fish in the Mediterranean. In addition, we investigated the molecular mechanism that determines the recovery of PFKL activity after refeeding previously starved fish.

MATERIALS AND METHODS

Enzyme Purification—The following steps were performed at 4°C unless otherwise stated. Frozen liver from *S. aurata* (80–100 g) was homogenized in 3 volumes of buffer H (50 mM Tris–HCl, pH 8, 50 mM K_2HPO_4, 50 mM KF, 4 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 0.5 mM benzamidin, 2 μg/ml antipain, 0.5 μg/ml leupeptin and 1 μg/ml aprotinin) using a Waring blender. The supernatant was collected after centrifugation of the homogenized sample (12,000*g*, 45 min), filtered through glass wool and submitted to cold acetone fractionation. The protein fraction precipitated between 35% and 50% saturation was recovered by centrifugation (12,000*g*, 15 min) and resuspended in 60–75 ml of buffer L (30 mM Tris–HCl, pH 8, 20 mM KF, 2 mM EDTA, 20% glycerol, 1 mM dithiothreitol, 0.5 mM PMSF, 0.5 mM benzamidin, 2 μg/ml antipain, 0.5 μg/ml leupeptin and 1 μg/ml aprotinin). The suspension obtained was heat-treated by incubation for 2 min in a 50°C water bath, followed by centrifugation for 15 min at 12,000*g*. The supernatant was fractionated with poly (ethylene glycol) 6,000 (Merck). The 5–12% pelleted fraction recovered after centrifugation (12,000*g*, 15 min) was resuspended in 25 ml of buffer L. This fraction was applied to an ATP-agarose column, previously equilibrated with buffer L, at a flow rate of 17 ml/h. After washing the column at 45 ml/h with buffer L, PFKL activity was eluted with a linear gradient of 0–10 mM fructose-6-phosphate and ADP in buffer G (30 mM Tris–HCl, pH 8, 30 mM K_2HPO_4, 20 mM KF, 2 mM EDTA, 20% glycerol, 1 mM dithiothreitol, 0.5 mM PMSF, 0.5 mM benzamidin, 2 μg/ml antipain, 0.5 μg/ml leupeptin and 1 μg/ml aprotinin). The eluted fractions were collected at a flow rate of 45 ml/h. The ATP-agarose fractions with PFKL activity were pooled, extensively dialysed against buffer L and loaded onto a DEAE-Sepharose Fast Flow column, equilibrated with buffer L. The column was washed with buffer L and fractions containing PFKL activity were eluted with a load of 250 mM K_2HPO_4 in buffer L. Flow rates during sample loading, washing and elution were as described above. The active fractions were pooled, dialysed and applied to a phosphocellulose column equilibrated in buffer L. Following washing with buffer L, PFKL was eluted with a 35–300 mM K_2HPO_4 linear gradient in buffer L. The PFKL-active fractions were pooled, dialysed and loaded onto a Blue-Sepharose column equilibrated with buffer L. The column was washed with buffer L and eluted with a linear gradient of 0–10 mM fructose-6-phosphate and ADP in buffer G. Fractions containing PFKL activity were pooled, extensively dialysed against buffer L and concentrated using Amicon provided with Diaflo (PM-10) filters. After addition of 30% glycerol, the enzyme solution was stored at −20°C in small portions.

Native Molecular Weight—Crude liver extracts from *S. aurata* were applied to a Bio-Gel A 1.5 m gel exclusion column (12 ml/h) equilibrated with buffer G and then eluted with the same buffer. Fractions containing PFKL were identified by measurement of enzyme activity and SDS–PAGE analysis. The native molecular weight of PFKL was calculated by comparison of its elution volume to the elution position of standard protein markers (thyroglobulin 669,000, apoferritin 443,000, alcohol dehydrogenase 150,000, seroalbumin 66,000 and carbonic anhydrase 29,000; Sigma) under identical chromatography conditions.

SDS–PAGE, Silver Staining and Western Blotting—SDS–PAGE was carried out according to the method of Laemmli (*21*). Samples for SDS–PAGE were incubated in SDS–PAGE sample buffer (62.5 mM Tris–HCl (pH 6.8) containing 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (w/v) glycerol and 0.02% (w/v) bromophenol blue) at 100°C for 5 min. Proteins in 10% polyacrylamide gels were visualized by Coomassie blue or silver staining (*22*). For Western blot analysis, proteins separated on 7% polyacrylamide gels were equilibrated in transfer buffer (25 mM Tris–HCl, pH 8.3, 192 mM glycine, 20% methanol) and electroeluted onto a Hybond-C super cellulose nitrate membrane (Amersham) at 60 V for 3 h at 4°C. The membranes were blocked with 10% non-fat dry milk in Tris-buffered saline and then incubated with a polyclonal anti-rat brain PFK (1 : 250; v/v) in 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20. Cross-reacting proteins were detected by use of a horseradish peroxidase-conjugated donkey anti-rabbit IgG (1 : 20,000; v/v) and an enhanced chemiluminescence kit (ECL Western Blotting, Amersham).

Enzyme Assays and Fru-2,6-P_2 Determination—PFK activity was measured using a Cobas Mira S spectrophotometric analyser (Hoffman-La Roche) at 30°C by monitoring the oxidation of NADH at 340 nm with coupled enzyme reactions (*16*). PFK activity was determined in a final volume of 200 μl containing: 100 mM Tris–HCl pH 8.25, 5 mM $MgCl_2$, 50 mM KCl, 0.15 mM NADH, 4 mM ammonium sulphate, 12 mM 2-mercaptoethanol, 10 mM fructose 6-phosphate, 30 mM glucose 6-phosphate, 0.675 U ml^{-1} aldolase, 5 U ml^{-1} triose phosphate isomerase, 2 U ml^{-1} glycerol 3-phosphate dehydrogenase and 4 μl of eluted fraction. PFK activity was monitored after addition of 1 mM ATP. For kinetic studies, assay mixtures containing substrates and effectors were performed as indicated in the legends of the corresponding figures. Kinetic parameters were calculated from 3 to 5 assays in at least 2 enzyme preparations. Curve fitting was carried out using Sigma plot software. A unit of enzyme activity is defined as the amount of enzyme producing 1 μmol of fru-1,6-P_2 per min (or consuming 2 μmol of NADH per minute).

Table 1. **Purification of PFKL from liver of *S. aurata*.**

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Crude extract	252	5,660	0.04	1.00	100
12% PEG precipitation	150	690	0.22	5.50	59.4
ATP-Agarose	103	26.3	3.94	98.5	41.0
DEAE-Sepharose	60.3	4.40	13.7	343	24.0
Phosphocellulose	21.8	0.19	115	2,870	8.66
Blue-Sepharose	12.5	0.02	627	15,700	4.97

Total protein content in the purification aliquots was determined by the method of Bradford (*23*) at 30°C using bovine serum albumin as a standard. Fru-2,6-P_2 was determined at 30°C on alkaline (NaOH, 50 mM) tissue extracts following an automated assay previously described (*24*).

Animals and Dietary Conditions—Gilthead sea bream fish, *Sparus aurata*, were obtained from Tinamenor (Cantabria, Spain) and were maintained in 250-l aquaria supplied with running sea-water at 20°C in a closed system with active pump filter and UV lamps. Photoperiod was regulated as a dark/light cycle of 12/12 h. To study the effect of nutritional status on regulation of PFKL activity and protein levels in the liver of *S. aurata*, fish were fed an experimental diet containing: 31.6% carbohydrates, 37.4% protein, 11.6% lipids, 16.0% ash, 3.4% moisture and 18.9 kJ/g of gross energy. Fish were fed at 2% body weight (BW) for 18 days, then starved for 19 days and finally allowed to refeed for 8 days with the same diet at 2% BW. To obtain liver samples, fish were anaesthetized with MS-222 (1:12,500) and killed by cervical section. The liver was dissected out, immediately frozen in liquid nitrogen and kept at −80°C until use. Crude extracts for assaying PFKL activity were obtained by homogenization of the powdered frozen liver (1/5, W/V) in 50 mM Tris–HCl pH 7.5, 4 mM EDTA, 50 mM NaF, 0.5 mM PMSF, 1 mM DTT and 250 mM sucrose using a PTA-7 Polytron (position 3, 30 s), and centrifugation at 20,000*g* for 30 min at 4°C.

RESULTS

Purification of PFKL from S. aurata—PFKL was purified following the method described by Su and Storey (*25*) with modifications. The following steps were performed: cold acetone fractionation, heat-treatment, poly (ethylene glycol) fractionation, ATP-agarose affinity chromatography, ion-exchange DEAE-Sepharose chromatography, and phosphocellulose and Blue-Sepharose chromatography. Table 1 shows the purification scheme and the mean values of four independent preparations. PFKL purification in the different steps was evaluated by SDS–PAGE (Fig. 1A). The elution profile of *S. aurata* PFKL was similar to that described for rainbow trout PFKL (*25*). The specific activity of purified *S. aurata* PFKL was 627 U/mg, a value higher than the specific activity described for the enzyme purified from other vertebrates (*25–27*). Purified *S. aurata* PFKL is composed of subunits with an apparent molecular weight of 86 kDa, as deduced from its relative mobility as a single polypeptide compared to the mobility of standard protein markers in SDS–PAGE experiments (Fig. 1A).

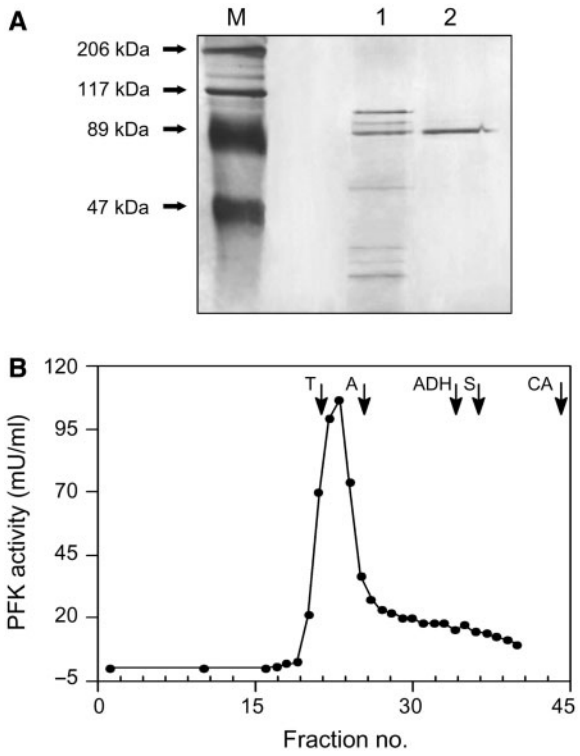

Fig. 1. **SDS–PAGE analysis during PFKL purification from *S. aurata*, and elution profile of PFKL activity in liver extracts.** (A) Proteins were separated on 10% polyacrylamide gels and silver stained. Lane 1, pooled PFKL fractions recovered after phosphocellulose chromatography; lane 2, eluted PFKL fractions recovered after Blue-sepharose chromatography; and lane M, standard protein markers. The molecular sizes of the markers are indicated in kilodaltons on the right side of the figure. (B) PFKL activity in crude liver extracts was assayed on the eluted fractions of a Bio-Gel A 1.5 m gel filtration column. The chromatographic process was performed as described in Materials and methods. The arrows indicate the relative elution fractions of standard protein markers (T, thyroglobulin; A, apoferritin; ADH, alcohol dehydrogenase; S, seroalbumin; CA, carbonic anhydrase).

The native molecular weight of *S. aurata* PFKL was estimated by comparing its elution profile through a Bio-Gel A 1.5 m gel filtration column with the elution of various standard proteins. Native PFKL eluted between thyroglobulin (669 kDa) and apoferritin (443 kDa) from the gel filtration column. The relative elution value calculated for PFKL was extrapolated from the standard plot to a molecular weight of ~510 kDa for the native enzyme (Fig. 1B). Altogether, these results suggest that native PFKL from *S. aurata* is a homohexamer.

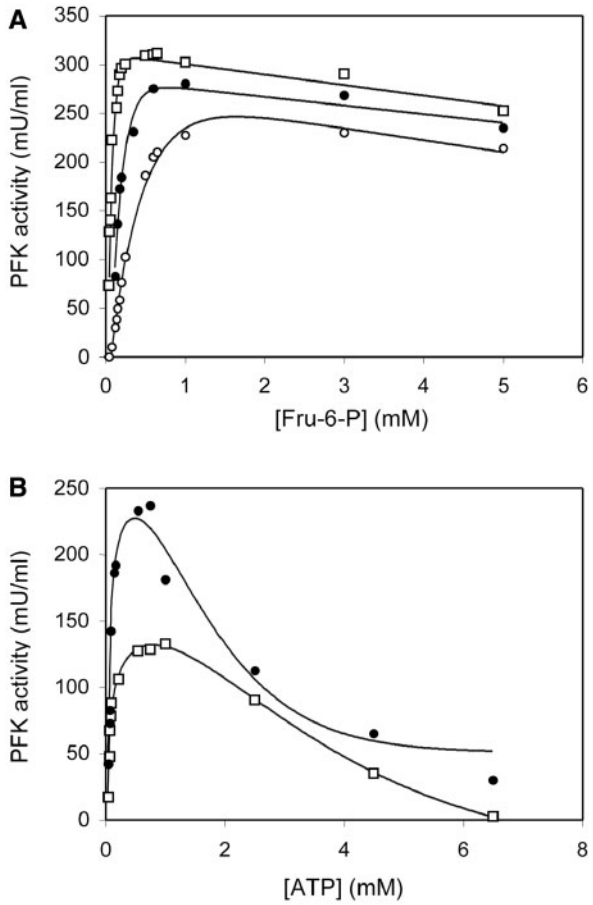

Fig. 2. **Effect of ATP on PFKL kinetics for fru-6-P, and of pH on kinetics for ATP.** (A) PFKL kinetics for fru-6-P. PFK activity was assayed by varying fru-6-P concentration at pH 7 in the presence of 0.5 mM (open square), 1 mM (filled circle) or 2.5 mM (open circle) ATP. (B) Effect of pH on PFKL kinetics for ATP. PFK activity was assayed by varying ATP concentration at 1 mM fru-6-P and at pH 7 (open square) or pH 8 (filled circle).

The effect of pH on the activity of *S. aurata* PFKL was studied at pH values between 7.25 and 9.5. Maximal activity was observed at pH 8–8.25.

Kinetic Properties of S. aurata PFKL—Kinetic studies of native PFKL were performed with enzyme purified to homogeneity from liver of *S. aurata*. At pH 7, saturation kinetic plots for fru-6-P (ranging from 0.01 to 5 mM) indicated sigmoidal behaviour (Fig. 2A). In the presence of 1 mM ATP, $S_{0.5}$ for fru-6-P was 0.034 mM. Hill plot analysis (log $[V/V_{max}\text{-}V]$ versus log[fru-6-P]) produced a linear function which enabled a Hill coefficient value of 1.7 to be calculated. Affinity of the enzyme for fru-6-P depended on ATP concentration: Increasing ATP from 0.5 to 2.5 mM increased 12-fold $S_{0.5}$ for fru-6-P. Hill coefficient and V_{max} were less affected by increased ATP concentrations; the former increased 1.3-fold and the latter decreased by about 26% (Table 2). Cooperativity binding of fru-6-P to PFKL showed dependence on pH: An increase in pH from 7 to 8 changed saturation kinetics for fru-6-P from sigmoidal to hyperbolic (Table 3).

The effect of ATP concentration on the activity of purified PFKL was also studied. ATP acts as a phosphate donor in the catalytic reaction and has two binding sites for PFK in mammals. At low concentrations, ATP binds the catalytic locus of PFK. However, high concentrations of ATP inhibit the enzyme by binding the nucleotide to an allosteric site (*28*). This effect was also observed for *S. aurata* PFKL when using a concentration of ATP above 1 mM (Fig. 2B): At pH 7 and in the presence of 1 mM fru-6-P, *S. aurata* PFKL displayed hyperbolic kinetics with a K_M for ATP of 0.051 mM and an apparent Hill coefficient of 0.98. Similar behaviour for ATP binding has been described for trout and rat PFK (*25*, *29*, *30*): Increasing pH from 7 to 8 raised V_{max}, whereas K_M for ATP was unaffected.

Table 2. **Effect of ATP concentration on PFKL kinetic parameters for fru-6-P.**

ATP (mM)	$S_{0.5}$ for fru-6-P (mM)	V_{max} (mU/ml)	n
0.50	0.010 ± 0.005	317 ± 10	1.49 ± 0.17
1.00	0.034 ± 0.005	278 ± 3	1.70 ± 0.05
1.50	0.062 ± 0.030	251 ± 9	1.91 ± 0.02
2.50	0.120 ± 0.029	235 ± 2	1.91 ± 0.10

The kinetic parameters were determined at pH 7 by varying fru-6-P concentration at fixed ATP levels. $S_{0.5}$ for fru-6-P is the concentration of fru-6-P required to give half-maximum velocity, V_{max} corresponds to the maximum velocity and n to the Hill coefficient.

Table 3. **Effect of pH on PFKL kinetics for fru-6-P.**

pH	$S_{0.5}$ for fru-6-P (mM)	V_{max} (mU/ml)	n
7	0.010 ± 0.005	139 ± 10	1.49 ± 0.17
8	0.047 ± 0.001	194 ± 8	1.00 ± 0.01

The kinetic parameters were determined at pH 7 and 8 by varying fru-6-P concentration at a fixed ATP concentration of 0.5 mM. $S_{0.5}$ for fru-6-P is the concentration of fru-6-P required to give half-maximum velocity, V_{max} corresponds to the maximum velocity and n to the Hill coefficient.

Allosteric Effectors of S. aurata *PFKL*—Fru-2,6-P_2 is the most potent activator of the majority of PFK described so far and an inhibitor of the gluconeogenic enzyme fructose-1,6-bisphosphatase. In addition, fru-2,6-P_2 counteracts ATP and citrate inhibition by increasing affinity for fru-6-P and reducing Hill coefficient (*31*). In the present study, the effect of fru-2,6-P_2 on PFKL purified from liver of *S. aurata* was studied at various concentrations of fru-6-P (Fig. 3A). In the absence of fru-2,6-P_2, saturation kinetics for fru-6-P were sigmoidal with a Hill coefficient of 1.91. However, the presence of 5 μM fru-2,6-P_2 resulted in hyperbolic behaviour of the enzyme and reduced 5-fold $S_{0.5}$ for fru-6-P. Concerning the effect of fru-2,6-P_2 on PFKL kinetics for ATP, the presence of 5 μM fru-2,6-P_2 caused modest effects on apparent K_M for ATP (1.5-fold increase) and V_{max} (9% decrease) (Table 4).

Adenine nucleotides such as AMP and ADP activate PFK from a variety of tissues and animals (*30*, *32–34*). In the present study, AMP and ADP modulated *S. aurata* PFKL but had opposite effects on the enzyme activity. At inhibitory concentrations of ATP (above 1 mM), 0.5 mM AMP partially neutralized ATP-mediated inhibition (Fig. 3B). However, ADP (0.5 mM) inhibited PFKL activity at all ATP concentrations tested (Fig. 3C). As other

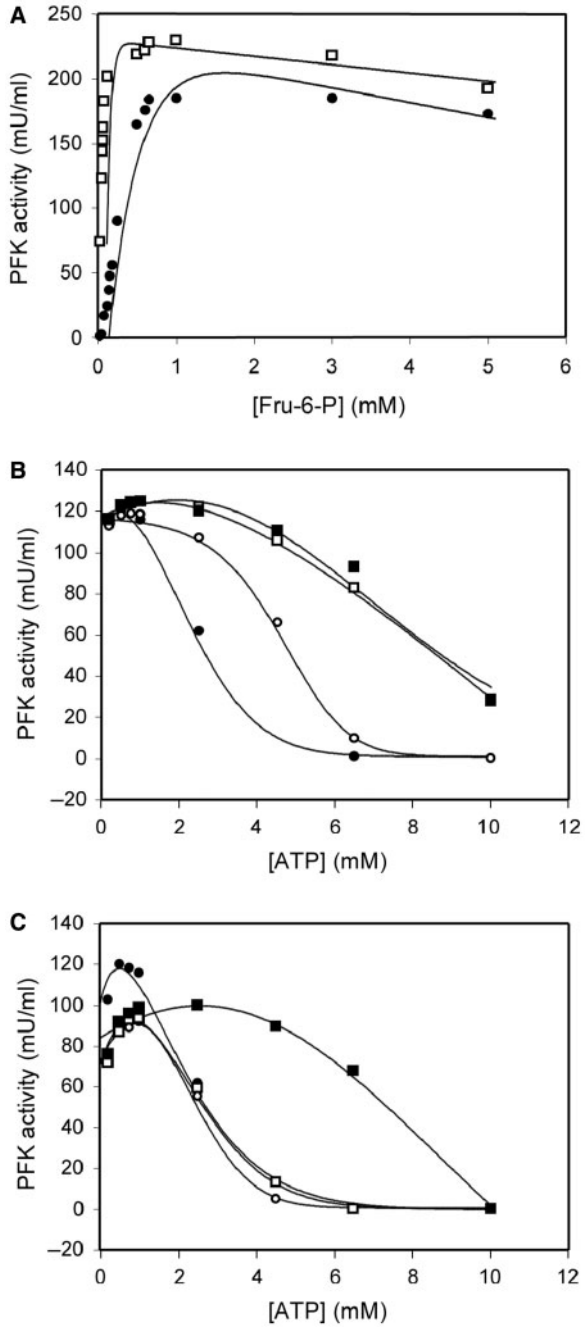

Fig. 3. **Effect of fru-2,6-P_2, AMP and ADP on *S. aurata* PFKL activity.** (A) Effect of fru-2,6-P_2 on PFKL kinetics for fru-6-P. PFK activity was assayed at pH 7 by varying fru-6-P concentration at 2.5 mM ATP in the presence (open square) or absence (filled circle) of 5 μM fru-2,6-P_2. (B) Effect of fru-2,6-P_2 on AMP-mediated activation of PFKL. Enzyme activity was assayed by varying ATP concentration at pH 7 and 0.6 mM fru-6-P in the absence (filled circle) or presence (open circle, open square, filled square) of 0.5 mM AMP. The effect of fru-2,6-P_2 on AMP-mediated activation was analysed by adding 1.5 μM (open square) or 5 μM (filled square) fru-2,6-P_2. (C) Effect of fru-2,6-P_2 on ADP-mediated inhibition of PFKL. Enzyme activity was assayed by varying ATP concentration at pH 7 and 0.6 mM fru-6-P in the absence (filled circle) or presence (open circle, open square, filled square) of 0.5 mM ADP. The effect of fru-2, 6-P_2 on ADP-mediated inhibition was analysed by adding 1.5 μM (open square) or 5 μM (filled square) fru-2,6-P_2.

Table 4. **Effect of fru-2,6-P_2 on PFKL kinetics for ATP.**

fru-2,6-P_2 (μM)	K_M for ATP (mM)	V_{max} (mU/ml)	n
0	0.051 ± 0.003	201 ± 12	1.00
5	0.076 ± 0.006	182 ± 4	1.00

The kinetic parameters were determined at pH 7 by varying ATP concentration at a fixed fru-6-P concentration of 1 mM. K_M for ATP is the concentration of ATP required to give half-maximum velocity, V_{max} corresponds to the maximum velocity and n to the Hill coefficient.

PFKs (*35*, *36*), *S. aurata* PFKL displayed a synergistic stimulation of enzyme activity by fru-2,6-P_2 and AMP: At 0.5 mM AMP and in the absence of fru-2,6-P_2, I_{50} for ATP was 4.74 mM. This effect was strengthened 1.66- and 1.72-fold by the addition of 1.5 and 5 μM fru-2,6-P_2, respectively. Regarding the inhibitory effect of ADP, a higher concentration of fru-2,6-P_2 (5 μM) was required to counteract the effect of 0.5 mM ADP. In addition, 5 μM fru-2,6-P_2 increased I_{50} for ATP 2.63-fold, whereas no significant change in I_{50} for ATP was observed in the absence of fru-2,6-P_2 or presence of 1.5 μM fru-2,6-P_2.

The effect of citrate, 3-phosphoglycerate (3-PGA), phosphoenolpyruvate (PEP), 6-phosphogluconate and phosphocreatine on *S. aurata* PFKL activity was also analysed. Since we previously found that these compounds are allosteric inhibitors of *S. aurata* muscle PFK (PFKM), we performed the assay by comparing their action on fish liver and muscle isozymes. In vertebrates, citrate is a potent inhibitor of PFKM, but exerts less or no effect on the liver isozyme in other animals (*26*, *29*, *37*). Similarly, citrate inhibited *S. aurata* PFKL to a lesser extent than in the muscle isozyme (Fig. 4A). At 0.5 mM ATP, I_{50} for citrate was 0.42 mM and 0.53 μM for PFKL and PFKM, respectively. ATP strengthened the effect of citrate on *S. aurata* PFKL (Fig. 4B): At 2.5 mM ATP, I_{50} for citrate was 0.42 mM, 9-fold lower than at 0.5 mM ATP (3.68 mM). Similarly as in rat PFK (*38*), at 0.6 mM fru-6-P and 1 mM ATP, 5 μM fru-2,6-P_2 totally neutralized citrate inhibition. At 2.5 mM ATP, fru-2,6-P_2 partially counteracted citrate inhibition and apparent I_{50} for citrate was 6 mM.

3-PGA is a glycolytic intermediate formed by transfer of a phosphate group from 1,3-bisphosphoglycerate to ADP. As in rabbits (*26*), 3-PGA had a modest inhibitory action on *S. aurata* PFKL, compared with its effect on the muscle isoform (Fig. 5A). At 0.5 mM ATP and 0.6 mM fru-6-P, I_{50} for 3-PGA was 0.5 and 3.9 mM for *S. aurata* PFKM and PFKL, respectively. As in other organisms, PEP, another glycolytic intermediate, inhibited *S. aurata* PFKM. However, it had only slight effects on PFKL: The I_{50} value for PEP was 5.13 mM. Thus, to inhibit *S. aurata* PFKL, a concentration 10-fold higher than the physiological content of PEP in liver was required (Fig. 5B). Neither 6-phosphogluconate nor phosphocreatine significantly affected *S. aurata* PFKL (Fig. 5C and D).

Nutritional Regulation of S. aurata PFKL—We previously demonstrated that starvation decreases the activity of *S. aurata* PFKL, whereas refeeding stimulates the enzyme activity (*16*, *19*, *39*). To determine whether changes in PFKL activity during starvation-refeeding resulted from modulation of protein levels or regulation

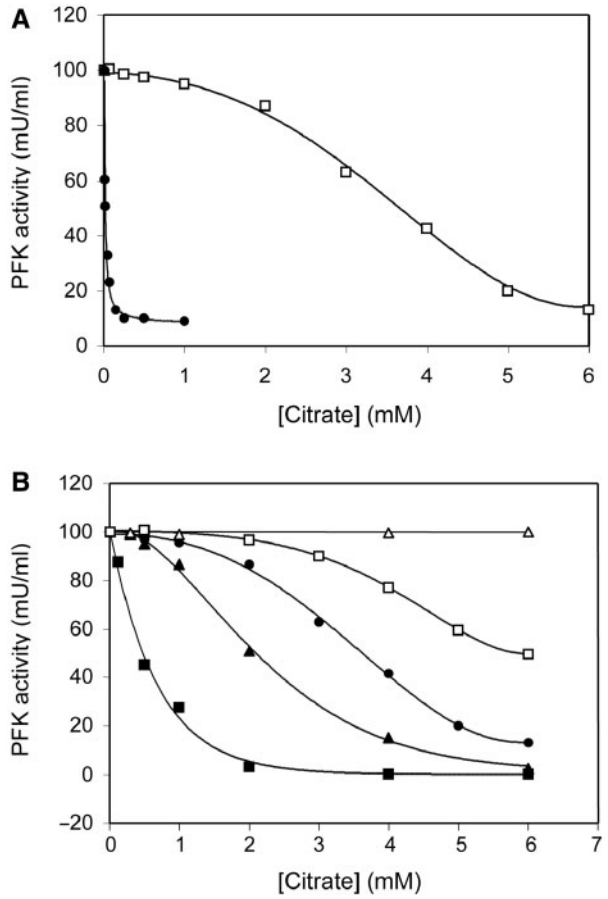

Fig. 4. **Inhibition of *S. aurata* PFKL by citrate.** (A) Effect of citrate on PFKL and PFKM isozymes. PFK activity was assayed by varying citrate concentration at pH 7 and at optimal concentrations of fru-6-P and ATP for liver (0.6 mM fru-6-P and 0.5 mM ATP; open square), and muscle (1 mM fru-6-P and 1 mM ATP; filled circle) enzymes. (B) Effect of ATP and fru-2,6-P_2 on inhibition of PFKL activity by citrate. PFK activity was assayed by varying citrate concentration at pH 7 and 0.6 mM fru-6-P in the presence of: 0.5 mM ATP and absence of fru-2,6-P_2 (filled circle); 1 mM ATP and absence of fru-2,6-P_2 (filled triangle); 1 mM ATP and 5 μM fru-2,6-P_2 (open triangle); 2.5 mM ATP and absence of fru-2,6-P_2 (filled square); or 2.5 mM ATP and 5 μM fru-2,6-P_2 (open squre).

of enzyme activity, we performed western blots and analysed PFKL activity in liver extracts of fed, 19-day starved and up to 8-day refed *S. aurata*. As shown in Fig. 6, starvation for 19 days caused a major decrease in both immunodetectable and PFKL activity. However, the time-course for recovery after refeeding differed for protein content and enzyme activity. While PFKL activity showed about 75% recovery in 1-day refed fish, refeeding up to 3 days did not significantly change the protein levels from those in starved animals. Long-term refeeding (8 days) restored both enzyme activity and protein levels.

As PFKL activity may be importantly affected by the hepatic content of fru-2,6-P_2, we also analysed fru-2,6-P_2 levels in the liver of starved and refed *S. aurata*. Consistently with previous results (*17*, *40*), starvation decreased hepatic fru-2,6-P_2 from 79 nmol/g of liver to very low levels (0.15 nmol/g of liver). A significant partial recovery of fru-2,6-P_2 was observed in the liver of 1-day refed fish (7.5 nmol/g of liver). Thereafter, 8 days of refeeding allowed fru-2,6-P_2 levels to gradually recover up to values similar to those observed in fed fish (Fig. 6).

DISCUSSION

In the present study, we addressed PFKL purification, kinetic characterization and changes in enzyme activity and protein content by starvation-refeeding in the liver of *S. aurata*. The enzyme was purified to homogeneity by acetone and poly (ethylene glycol) 6,000 fractionation followed by four different chromatographic steps. The estimated subunit molecular mass was 86 kDa. Thus, the molecular weight of PFKL subunit is slightly higher than that in rat liver [83 kDa; (*41*)] and trout [76 kDa; (*25*)], but similar to bovine parotid gland [86 kDa; (*42*)]. The calculated molecular mass of the native enzyme was 510 kDa, which indicates homohexameric structure for *S. aurata* PFKL. Although the polymerization structure of most PFKs is tetrameric, hexameric configuration of PFKL was also described for rainbow trout muscle (*43*).

As in rabbits (*26*) and unlike in rainbow trout (*25*, *29*), *S. aurata* PFKL exhibited lower affinity for fru-6-P and higher cooperativity than the muscle isozyme isolated from the same species (*34*). This is consistent with the major role of gluconeogenesis in the liver and of glycolysis in skeletal muscle, where PFKM allows rapid increases in glucose utilization for short periods of time. Indeed, the specific activity of *S. aurata* PFKL (627 U/mg) is considerably lower than the value that we previously observed for the isozyme isolated from skeletal muscle (2,080 U/mg) (*34*). Unlike the muscle enzyme, and as in trout and rat, saturation kinetics for ATP of *S. aurata* PFKL followed hyperbolic behaviour. Since K_M for ATP was not significantly affected by pH or fru-2,6-P_2, the residues involved in ATP binding may reside in a domain of the enzyme that is not significantly affected during allosteric transition. The effect of ATP concentration on $S_{0.5}$ for fru-6-P was stronger for the *S. aurata* liver isozyme than for PFKM (*34*), suggesting that PFKL is more susceptible to the inhibitory effect of ATP than the muscle enzyme. This effect may be related to the fact that lower levels of ATP are found in liver than in muscle (*25*, *29*).

As in mammals, fru-2,6-P_2 behaved as a potent allosteric activator of *S. aurata* PFKL. The activating effect of fru-2,6-P_2 on *S. aurata* PFKL resulted from increased affinity for fru-6-P and reduced cooperativity. In addition, fru-2,6-P_2 operated synergistically with AMP and counteracted the inhibitory action of ATP, ADP and citrate. In a physiological condition where fru-2,6-P_2 levels are low, changes in fru-6-P may play a major role in the regulation of *S. aurata* PFKL activity. Besides, kinetics for fru-6-P showed sigmoidal behaviour and $S_{0.5}$ for fru-6-P was 0.27 mM, a value that lies within the range of the hepatic content of fru-6-P in fed *S. aurata* [about 0.2–0.4 mM; (*39*)]. However, the regulatory role of fru-6-P disappears in the presence of 5 μM fru-2,6-P_2, which reduced 5-fold $S_{0.5}$ for fru-6-P and converted saturation curves for fru-6-P to hyperbolic. Furthermore,

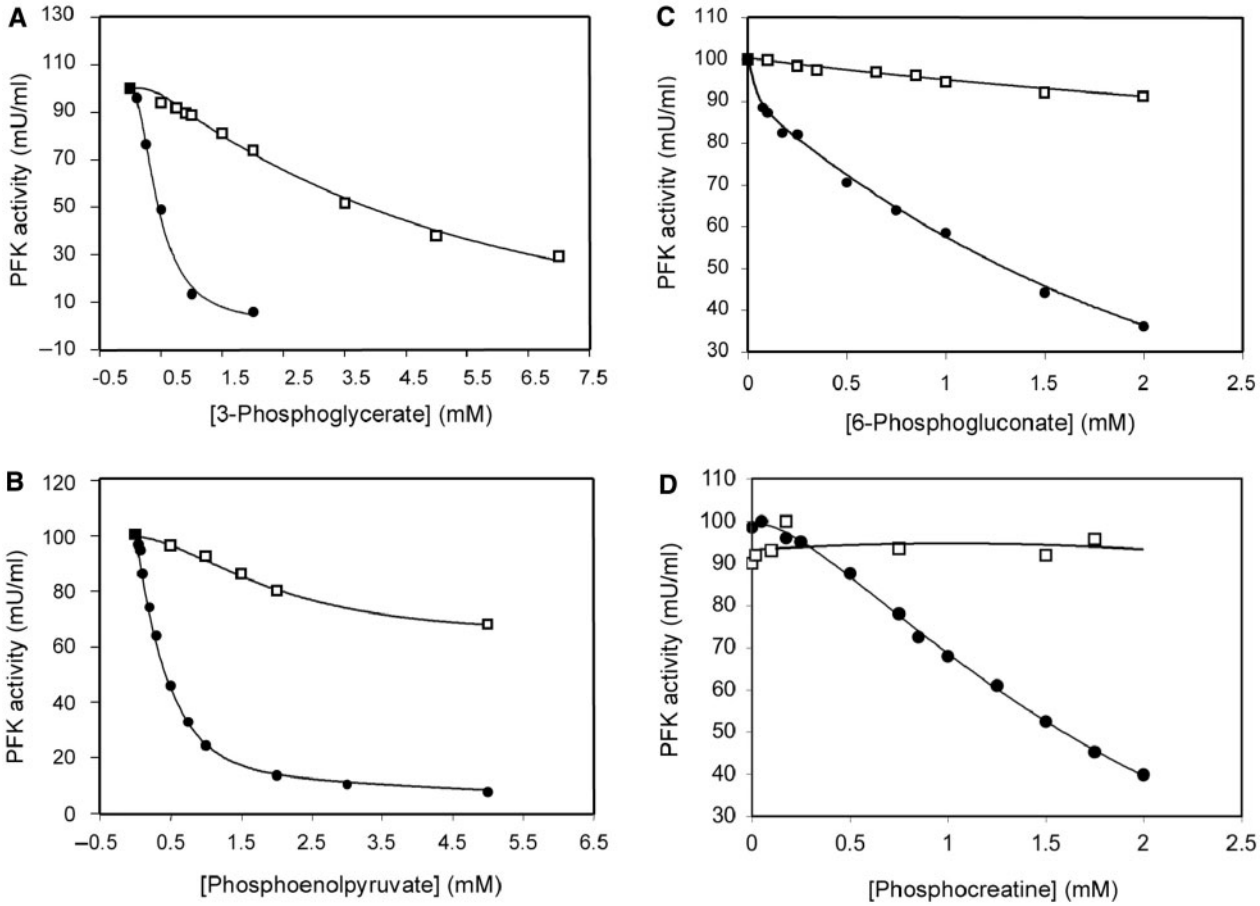

Fig. 5. **Inhibition of *S. aurata* PFKL and PFKM by 3-PGA, PEP, 6-phosphogluconate and phosphocreatine.** (A) Effect of 3-PGA. PFK activity was assayed by varying 3-PGA concentration at pH 7 and at optimal concentrations of fru-6-P and ATP for liver (0.6 mM fru-6-P and 0.5 mM ATP; open squre) and muscle (1 mM fru-6-P and 1 mM ATP; filled circle) enzymes. (B) Effect of PEP. PFK activity was assayed by varying PEP concentration at pH 7 and at optimal concentrations of fru-6-P and ATP for liver (0.6 mM fru-6-P and 0.5 mM ATP; open square) and muscle (1 mM fru-6-P and 1 mM ATP; filled circle) enzymes. (C) Effect of 6-phosphogluconate. PFK activity was assayed by varying 6-phosphogluconate concentration at pH 7 and at optimal concentrations of fru-6-P and ATP for liver (0.6 mM fru-6-P and 0.5 mM ATP; open square) and muscle (1 mM fru-6-P and 1 mM ATP; filled cirlce) enzymes. (D) Effect of phosphocreatine. PFK activity was assayed by varying phosphocreatine concentration at pH 7 and at optimal concentrations of fru-6-P and ATP for liver (0.6 mM fru-6-P and 0.5 mM ATP; open square) and muscle (1 mM fru-6-P and 1 mM ATP; filled circle) enzymes.

in *S. aurata* fed on high carbohydrate diets, the hepatic levels of fru-2,6-P_2 rises up to 90 μM (*17*, *20*, *40*). Under these conditions, PFKL may be saturated by fru-2,6-P_2 *in vivo*, which is consistent with the highly active glycolytic flux observed in the liver of *S. aurata* fed high carbohydrate diets (*16*, *17*, *40*).

Since PFKL was more sensitive to the inhibitory action of ATP, and was less affected by AMP or ADP than PFKM (*34*), it seems that control of PFK activity by AMP or ADP is more relevant in muscle than in liver. This behaviour may be related to the need for anaerobic production of energy in muscle. As in other species, citrate inhibited *S. aurata* PFKL to a lesser extent than the muscle isozyme. Furthermore, in the presence of fru-2,6-P_2, the concentration of citrate needed to inhibit *S. aurata* PFKL seems too high to be considered of physiological relevance in the piscine liver. Nevertheless, we cannot rule out citrate playing a role in the regulation of PFKL activity under nutritional conditions that induce low levels of fru-2,6-P_2 in the piscine liver, such as feeding high protein/low carbohydrate diets. In fact, as well as inhibiting PFKL, citrate inhibits 6-phosphofructo-2-kinase in *S. aurata* (*44*), which suggests that this metabolite can exert a dual effect to down-regulate glycolysis under specific physiological conditions.

The piscine liver isozyme was less sensitive to the action of 3-PGA, PEP, 6-phosphogluconate and phosphocreatine than *S. aurata* PFKM (*34*). Possibly, since the muscle cell changes deeply through periods of relaxation, intensive exercise or muscle contraction, flux of glycolysis in this tissue needs to be more finely regulated by salts and other effectors such as phosphocreatine than in the liver. The inhibitory action of 3-PGA on PFKL would avoid glycolysis when the energy demand in the cell is covered. Due to the high I_{50} for PEP shown by PFKL, it does not seem that this metabolite exerts an important regulatory role on PFKL activity in the liver. The fact that *S. aurata* PFKM was more sensitive to PEP inhibition is

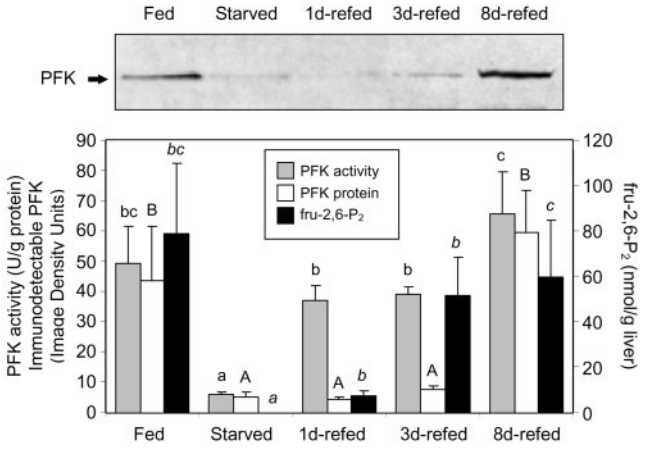

Fig. 6. **Effect of starvation and refeeding on PFKL protein, PFKL activity and fru-2,6-P_2 levels in the liver of *S. aurata*.** The upper panel shows a representative western blot of immunodetectable PFKL in liver of fed, 19-day starved, 1-day refed, 3-day refed and 8-day refed fish. The lower panel shows PFKL enzyme activity, immunodetectable PFKL and fru-2,6-P_2 levels determined in liver extracts of fed, 19-day starved, 1-day refed, 3-day refed and 8-day refed fish. PFKL activity, immunodetectable protein and fru-2,6-P_2 levels are indicated in U/g protein, Image Density Units and nmol/g liver, respectively, ± SD from determinations in 3–5 fish. Different letters (lower case for PFKL activity, capital for immunodetectable PFKL protein and italic letters for fru-2,6-P_2 levels) indicate significant differences according to Duncan's multiple range test among feeding conditions ($P < 0.05$).

consistent with previous observations in mammals (*26*, *45*) and may be related to a retroinhibitory effect of PEP on glycolysis in skeletal muscle, an essentially glycolytic tissue. Lack of regulation of PFKL and thus glycolysis by intermediates from the pentose phosphate pathway such as 6-phosphogluconate, may be related to the marked lipogenic function of the fish liver.

In mammals, it is well known that fasting and diabetes decrease PFKL activity, whereas refeeding high carbohydrate diets or insulin treatment restores it. Nutritional and hormonal regulation of PFKL expression differs between rats and mice. While in rats it seems to occur at the translational and posttranslational level (*1*, *2*, *5*), in mice the levels of PFKL mRNA are also controlled by fasting-refeeding and insulin (*3*, *4*). We previously showed that the activity levels of PFKL can be used as indicator of nutritional condition and growth performance of *S. aurata*. Starvation and caloric restriction decrease PFKL activity, whereas refeeding and supply of high carbohydrate/low protein diets stimulate the enzyme activity in the piscine liver (*16*, *19*, *39*). In the present study, analysis of PFKL activity and protein levels in the liver of *S. aurata* subjected to starvation and refeeding with a high carbohydrate diet, indicated that nutritional regulation of PFKL resulted from modulation of both enzyme activity and protein levels. Starvation promoted a significant decrease in immunodetectable and enzyme activity levels of PFKL. This is consistent with previous results of our group indicating metabolic adaptation of *S. aurata* liver to long-term starvation through a dramatic decrease in fru-2,6-P_2 levels and the activity of 6-phosphofructo-2-kinase and glycolytic regulatory enzymes, such as PFKL (*16*, *19*, *40*, *46*). The time-course followed by PFKL activity and protein content after refeeding suggests that short-term recovery of the enzyme activity (up to 3 days) may result from posttranslational regulation, rather than changes in protein levels. Consistently, 1 day of refeeding allowed fru-2,6-P_2 levels to increase from 0.15 nmol/g of liver (equivalent to 0.3 μM) in starved animals to 7.5 nmol/g of liver (equivalent to 15 μM), which is a concentration high enough to strongly activate PFKL, and thus may be responsible for the short-term activation of the enzyme. However, long-term regulation of PFKL in 8-day refed fish may result from the combination of allosteric effects of fru-2,6-P_2 and increased PFKL protein.

In conclusion, we purified and kinetically characterized PFKL from *S. aurata*. Unlike other vertebrate PFKs, *S. aurata* PFKL is active as a homohexamer and is inhibited by ADP. Compared to the *S. aurata* muscle isozyme, PFKL shows lower affinity for fru-6-P, higher cooperativity, hyperbolic kinetics for ATP, it is more susceptible to inhibition by ATP and less affected by AMP, ADP and inhibition by 3-PGA, PEP, 6-phosphogluconate and phosphocreatine. Given the strong activating effect of fru-2,6-P_2 on *S. aurata* PFKL, our findings indicate that piscine PFKL seems to be highly dependent on the fru-2,6-P_2 and ATP content of the cell. Increased fru-2,6-P_2 levels explain short-term recovery of PFKL activity after refeeding, while both activation by fru-2,6-P_2 and increased PFKL protein determine long-term restoration of PFKL activity. Knowledge of the effect of diet composition on kinetic properties of piscine PFKL will be useful to clarify to what extent it is possible to substitute protein by carbohydrate in diets supplied to fish in culture, which in turn will reduce amino acid degradation, loss of nitrogen to ambient waters and risk of eutrophication in intensive fish rearing.

This work was supported by the MCYT (Spain) grants BMC2000-0761 and BIO2003-01098. We thank Dr G. A. Dunaway (Department of Pharmacology, Southern Illinois University School of Medicine, Springfield, USA) for providing the polyclonal anti-rat brain PFK used in the present study and the Language Advisory Service of the University of Barcelona for correcting the English manuscript.

REFERENCES

1. Dunaway, G.A. Jr. and Weber, G. (1974) Effects of hormonal and nutritional changes on rates of synthesis and degradation of hepatic phosphofructokinase isozymes. *Arch. Biochem. Biophys.* **162**, 629–637
2. Dunaway, G.A., Leung, G.L., Thrasher, J.R., and Cooper, M.D. (1978) Turnover of hepatic phosphofructokinase in normal and diabetic rats. Role of insulin and peptide stabilizing factor. *J. Biol. Chem.* **253**, 7460–7463
3. Gehnrich, S.C., Gekakis, N., and Sul, H.S. (1988) Liver (B-type) phosphofructokinase mRNA. Cloning, structure, and expression. *J. Biol. Chem.* **263**, 11755–11759
4. Rongnoparut, P., Verdon, C.P., Gehnrich, S.C., and Sul, H.S. (1991) Isolation and characterization of the transcriptionally regulated mouse liver (B-type) phosphofructokinase gene and its promoter. *J. Biol. Chem.* **266**, 8086–8091
5. Hotta, K., Nakajima, H., Yamasaki, T., Hamaguchi, T., Kuwajima, M., Noguchi, T., Tanaka, T., Kono, N., and

Tarui, S. (1991) Rat-liver-type phosphofructokinase mRNA. Structure, tissue distribution and regulation. *Eur. J. Biochem.* **202**, 293–298
6. Poorman, R.A., Randolph, A., Kemp, R.G., and Heinrikson, R.L. (1984) Evolution of phosphofructokinase–gene duplication and creation of new effector sites. *Nature* **309**, 467–469
7. Brand, I.A. and Soling, H.D. (1974) Rat liver phosphofructokinase. Purification and characterization of its reaction mechanism. *J. Biol. Chem.* **249**, 7824–7831
8. Kemp, R.G. and Foe, L.G. (1983) Allosteric regulatory properties of muscle phosphofructokinase. *Mol. Cell. Biochem.* **57**, 147–154
9. Kemp, R.G. and Gunasekera, D. (2002) Evolution of the allosteric ligand sites of mammalian phosphofructo-1-kinase. *Biochemistry* **41**, 9426–9430
10. Dunaway, G.A., Kasten, T.P., Sebo, T., and Trapp, R. (1988) Analysis of the phosphofructokinase subunits and isoenzymes in human tissues. *Biochem. J.* **251**, 677–683
11. Reuter, R., Naumann, M., Bar, J., Haferburg, D., and Kopperschlager, G. (2000) Purification, molecular and kinetic characterization of phosphofructokinase-1 from the yeast Schizosaccharomyces pombe: evidence for an unusual subunit composition. *Yeast* **16**, 1273–1285
12. Domenech, C.E., Mieskes, G., and Soling, H.D. (1988) Does rat liver 6-phosphofructo-1-kinase exhibit 'half-of-the-sites-phosphorylation'? *Eur. J. Biochem.* **173**, 79–84
13. Claus, T.H., Schlumpf, J.R., El-Maghrabi, M.R., and Pilkis, S.J. (1982) Regulation of the phosphorylation and activity of 6-phosphofructo 1-kinase in isolated hepatocytes by alpha-glycerolphosphate and fructose 2,6-bisphosphate. *J. Biol. Chem.* **257**, 7541–7548
14. Metón, I., Egea, M., and Baanante, I.V. (2003) New insights into the regulation of hepatic glucose metabolism in fish in *Recent Research Developments in Biochemistry* (Pandalai, S.G., ed.) Vol. 4, pp. 125–149, Research Signpost, Kerala, India.
15. Wright, J.R. Jr., O'Hali, W., Yang, H., Han, X.X., and Bonen, A. (1998) GLUT-4 Deficiency and severe peripheral resistance to insulin in the teleost fish tilapia. *Gen. Comp. Endocrinol.* **111**, 20–27
16. Metón, I., Mediavilla, D., Caseras, A., Cantó, E., Fernández, F., and Baanante, I.V. (1999) Effect of diet composition and ration size on key enzyme activities of glycolysis-gluconeogenesis, the pentose phosphate pathway and amino acid metabolism in liver of gilthead sea bream (Sparus aurata). *Br. J. Nutr.* **82**, 223–232
17. Metón, I., Caseras, A., Fernández, F., and Baanante, I.V. (2000) 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene expression is regulated by diet composition and ration size in liver of gilthead sea bream, Sparus aurata. *Biochim. Biophys. Acta* **1491**, 220–228
18. Caseras, A., Metón, I., Vives, C., Egea, M., Fernández, F., and Baanante, I.V. (2002) Nutritional regulation of glucose-6-phosphatase gene expression in liver of the gilthead sea bream (Sparus aurata). *Br. J. Nutr.* **88**, 607–614
19. Metón, I., Fernández, F., and Baanante, I.V. (2003) Short- and long-term effects of refeeding on key enzyme activities in glycolysis-gluconeogenesis in the liver of gilthead sea bream (Sparus aurata). *Aquaculture* **225**, 99–107
20. García de Frutos, P., Bonamusa, L., Fernández, F., and Baanante, I.V. (1990) Fructose 2,6-bisphosphate in liver of Sparus aurata: influence of nutritional state. *Comp. Biochem. Physiol. B* **96**, 63–65
21. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
22. Morrissey, J.H. (1981) Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal. Biochem.* **117**, 307–310
23. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
24. Metón, I., Mediavilla, D., and Baanante, I.V. (1995) Automated assay for fructose 2,6-bisphosphate. *Anal. Lett.* **28**, 1919–1935
25. Su, J.Y. and Storey, K.B. (1993) Phosphofructokinase from liver of the rainbow trout, Oncorhynchus mykiss. *Arch. Biochem. Biophys.* **302**, 49–55
26. Kemp, R.G. (1971) Rabbit liver phosphofructokinase. Comparison of some properties with those of muscle phosphofructokinase. *J. Biol. Chem.* **246**, 245–252
27. Kono, N. and Uyeda, K. (1973) Chicken liver phosphofructokinase. II. Cold inactivation. *J. Biol. Chem.* **248**, 8603–8609
28. Uyeda, K. (1979) Phosphofructokinase. *Adv. Enzymol. Relat. Areas Mol. Biol.* **48**, 193–244
29. Su, J.Y. and Storey, K.B. (1994) Regulation of phosphofructokinase from muscle and liver of rainbow trout by protein phosphorylation. *Biochem. Mol. Biol. Int.* **33**, 1191–1200
30. Sola, M.M., Salto, R., Oliver, F.J., Gutierrez, M., and Vargas, A.M. (1993) Regulation of rat-renal cortex phosphofructokinase activity by pH. *Enzyme Protein* **47**, 99–104
31. Van Schaftingen, E., Jett, M.F., Hue, L., and Hers, H.G. (1981) Control of liver 6-phosphofructokinase by fructose 2,6-bisphosphate and other effectors. *Proc. Natl Acad. Sci. USA* **78**, 3483–3486
32. Uyeda, K., Furuya, E., and Luby, L.J. (1981) The effect of natural and synthetic D-fructose 2,6-bisphosphate on the regulatory kinetic properties of liver and muscle phosphofructokinases. *J. Biol. Chem.* **256**, 8394–8399
33. Villamarín, J.A. and Rodríguez-Torres, A.M. (1990) Effect of fructose 2,6-bisphosphate and ammonium ions on AMP-mediated activation of phosphofructokinase from the posterior adductor muscle of the sea mussel Mytilus galloprovincialis. *Mar. Biol.* **106**, 235–238
34. Mediavilla, D., Metón, I., and Baanante, I.V. (2007) Purification and kinetic properties of 6-phosphofructo-1-kinase from gilthead sea bream muscle. BBA-Gen. Subj. **1770**, 706–715.
35. Arts, E., Kubicek, C.P., and Rörh, M. (1987) Regulation of phosphofructokinase from Aspergillus niger: Effect of fructose 2,6-bisphosphate on the action of citrate, ammonium ions and AMP. *J. Gen. Microbiol.* **133**, 1195–1199
36. Espinet, C., Bartrons, R., and Carreras, J. (1988) Effects of fructose 2,6-bisphosphate and glucose 1,6-bisphosphate on phosphofructokinase from chicken erythrocytes. *Comp. Biochem. Physiol. B* **90**, 453–457
37. Andrés, V., Carreras, J., and Cussó, R. (1990) Regulation of muscle phosphofructokinase by physiological concentrations of bisphosphorylated hexoses: effect of alkalinization. *Biochem. Biophys. Res. Commun.* **172**, 328–334
38. Narabayashi, H., Lawson, J.W., and Uyeda, K. (1985) Regulation of phosphofructokinase in perfused rat heart. Requirement for fructose 2,6-bisphosphate and a covalent modification. *J. Biol. Chem.* **260**, 9750–9758
39. Bonamusa, L., García de Frutos, P., Fernández, F., and Baanante, I.V. (1992) Nutritional effects on key glycolytic-gluconeogenic enzyme activities and metabolite levels in the liver of the teleost fish Sparus aurata. *Mol. Mar. Biol. Biotech.* **1**, 113–125
40. Metón, I., Caseras, A., Mediavilla, D., Fernández, F., and Baanante, I.V. (1999) Molecular cloning of a cDNA encoding 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase from liver of Sparus aurata: nutritional regulation of enzyme expression. *Biochim. Biophys. Acta* **1444**, 153–165
41. Pilkis, S.J., El-Maghrabi, M.R., Pilkis, J., and Claus, T.H. (1982) Studies on the in vitro phosphorylation of

6-phosphofructo-1-kinase from rat liver. *Arch. Biochem. Biophys.* **215**, 379–389
42. Fukushima, E. and Sugiya, H. (1992) Purification and characterization of phosphofructokinase in bovine parotid gland. *Int. J. Biochem.* **24**, 1307–1314
43. Su, 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
44. García de Frutos, P. and Baanante, I.V. (1994) 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase in liver of the teleost Sparus aurata. *Arch. Biochem. Biophys.* **308**, 461–468
45. Tsai, M.Y. and Kemp, R.G. (1974) Rabbit brain phosphofructokinase. Comparison of regulatory properties with those of other phosphofructokinase isozymes. *J. Biol. Chem.* **249**, 6590–6596
46. Caseras, A., Metón, I., Fernández, F., and Baanante, I.V. (2000) Glucokinase gene expression is nutritionally regulated in liver of gilthead sea bream (Sparus aurata). *BBA-Gene Struct. Expr.* **1493**, 135–141