REVISTA ESPAÑOLA DE FISIOLOGIA, 44 (4), 381-386, 1988

Metabolic Regulation of Glycolysis in Sea Bass (*Dicentrarchus labrax* L.) Muscle. I. Kinetic Study and Characteristic Modulators of Pyruvate Kinase

M. D. Fideu*, M. L. Maroto, M. C. Serradilla; M. L. Pérez, M. J. Herranz and M. Ruiz-Amil**

Departamento de Bioquímica
Facultad de Veterinaria
Universidad Complutense
28040 Madrid

(Received on September 21, 1987)

M.D. FIDEU, M.L. MAROTO, M.C. SERRADILLA, M.L. PEREZ, M.J. HERRANZ and M. RUIZ-AMIL. *Metabolic Regulation of Glycolysis in Sea Bass (*Dicentrarchus labrax L.*) Muscle. I. Kinetic Study and Characteristic Modulators of Pyruvate Kinase.* Rev. esp. Fisiol., 44 (4), 381-386, 1988.

White muscle pyruvate kinase from sea bass presents positive cooperativity with respect to PEP substrate. The enzyme is regulated by F-1.6-P_2 and L-Phenylalanine. The activator effect of F-1.6-P_2 in experiments carried out for the substrate PEP with crude extract seems to indicate that the enzyme is activated *in vivo* by this compound. The enzyme was not inhibited by either alanine or ATP but was inhibited by L-phenylalanine. Therefore this enzyme presents kinetic and regulatory properties similar to those of the mammalian isozyme M_2.

Key words: Sea bass, White muscle, Pyruvate kinase.

The metabolic flow of cellular metabolism is controlled by the modulation of the activity and concentration of regulatory enzymes.

ATP: pyruvate phosphotransferase (E.C. 2.7.1.40) (PK) is widely described as a key enzyme of glycolysis in many organisms (8, 21, 25). It catalyses the reaction between phosphoenolpyruvate (PEP) and adenosine diphosphate (ADP) yielding pyruvate and adenosine triphosphate (ATP). Both compounds are located in a nodal point of metabolism, since phosphoenolpyruvate is the first substrate in the anaplerotic pathway of the Krebs cycle from glycolysis, and it is also a precursor for aromatic amino acids. Moreover, pyruvate can be oxidized via acetyl-CoA through the Krebs cycle or act as a precursor for amino acids clasified as «pyruvate group», pantothenic acid or lactate. Therefore, the regulation of pyruvate kinase activity could have a major physiological importance controlling the intracellular levels of phosphoenolpyruvate

* To whom correspondence should be addressed.

** Present address: Departamento de Bioquímica. Facultad de Farmacia. Universidad Complutense. 28040 Madrid.

and pyruvate and channelling the fluxes of these compounds to distinct pathways according to the cell needs: catabolic, anaplerotic or anabolic.

In general, the most significant modulators of PK activity are phosphorylated hexoses (14, 24), adenine nucleotides (3, 4) and amino acids (11). PK exists in isozymatic forms in different animal species. Considering only their kinetic behaviour, the majority of authors coincide on the existence of four fundamental types of PK in mammals (4, 10, 16). These four types of isozyme have also been described in fish (8, 9, 13, 19).

The results obtained in liver PK of *Dicentrarchus labrax* L. show that the enzyme presents kinetic and regulating properties similar to those of class L described in mammals (5). Nevertheless, the positive cooperativity of sea bass liver enzyme for the substrate PEP is not very accused, which can be considered a common characteristic in the mammal type M_2 enzyme.

In the present study, some enzymatic characteristics of PK from sea bass muscle have been determined. The kinetic behaviour with respect to PEP and ADP and the effect of alanine, phenylalanine, ATP and F-1.6-P_2 at 22° C has been studied. The purpose of this investigation was to determine the types of isozyme to which this PK belongs and to compare its behaviour to that of other tissues studied in this and other species of fish. We have also compiled supporting data for a better understanding of glycolysis regulation in fish.

Materials and Methods

Biological material. — Sea bass, *Dicentrarchus labrax* L., (weight 50 ± 10 g) were obtained from «Salina Esperanza Siglo XIX» (Cádiz, Spain) where they were maintained with a controlled diet and optimal oxygenation and salinity conditions.

The temperature varied with the season (10-28° C).

The muscles from the area between the tail and anal fins were dissected and frozen for no more than 10 days until use.

Homogenate preparation. — The enzyme extracts were obtained by homogenizing a previously weighed muscle sample with the appropriate volume of 50 mM Tris-maleic buffer pH 7.4 (maximum activity), containing glycerol 30 % (v/v), maintaining the proportion of 1 g of fresh tissue to 19 ml buffer. The homogenation was carried out at a temperature between 0° C and 4° C in an ice bath. Homogenates were centrifugued for 30 min at 26,000 × *g*. The supernatant layers were used for the estimation of the enzymatic activity. Glycerol was employed to maintain the enzymatic activity (at a concentration previously demonstrated not to affect this value). The homogenate in the presence of glycerol and then frozen at -20 ° C maintained 90 % of the PK activity for 168 hours.

Enzyme assays. — PK activity was assayed by the estimation of the NADH oxidation in a coupled system with lactate dehydrogenase, according to BERGMEYER (2). The reaction mixture contained in a final volume of 1 ml was: Tris-HCl 100 mM; PEP 1 mM; $MgCl_2$ 8 mM; KCl 75 mM; ADP 2 mM; NADH 0.2 mM; LDH 18 UI; enzyme extract 10 µl (containing 68 µg of protein). The reaction was started by adding PEP.

Enzyme units are expressed as µmole of substrate utilized, or product formed per minute.

For the study of the effect of ATP on the PK enzymatic activity, an equal concentration of Mg^{2+} was added with the aim of retaining this ion in the reaction mixture. All experiments were carried out with extract parcially purified with Sephacryl S-300.

All measurements were carried out at

22°C due to the importance of the temperature effect in the biochemical studies in organisms such as *D. labrax* L. (5, 6). Proteins were determined by the method of LOWRY *et al.* (15).

Chemical and reagents. — Phosphoenolpyruvate (PEP), adenosine-5-diphosphate (ADP) nicotinamide adenine dinucleotide reduced form (NADH) were from Boehringer-Mannheim. Other reagents of analytic grade were purchased from Merck Darmstad.

Results and Discussion

Figure 1 shows the curve of velocity versus the substrate concentration.

The reaction rate is not hyperbolic with respect to the substrate concentration of PEP as can be deduced from the Lineweaver-Burk (not shown) and Hill plots (graph A). The values of Hill coefficient and $K_{0.5}$ were 1.9 and 0.35 mM respectively. The other substrate ADP did not show a cooperativity effect (graph B) and the K_m value estimated from Lineweaver-Burk plot (not shown) was 0.25 mM.

Fructose-1.6-biphosphate (F-1.6-P_2) is a known activator of PK in many organisms and in different fish tissue (1, 5, 7, 19, 20, 26), is particularly so in the muscle enzyme of goldfish (13) and other teleost (17, 22).

F-1.6-P_2 activates sea bass, muscle PK and changes the sigmoidal behaviour for PEP, giving rise to a Michaelian Kinetic (figure 2). The Hill coefficient and Km values are 1.1 and 0.34 mM respectively.

The study was carried out with 0.01 mM of F-1.6-P_2, closer to the physiological concentration in fishes, that produces the maximum activation; moroever, the experiments performed with the crude extract displayed a hyperbolic kinetic and did not show F-1.6-P_2 activation, which could indicate that this enzyme is activated *in vivo*.

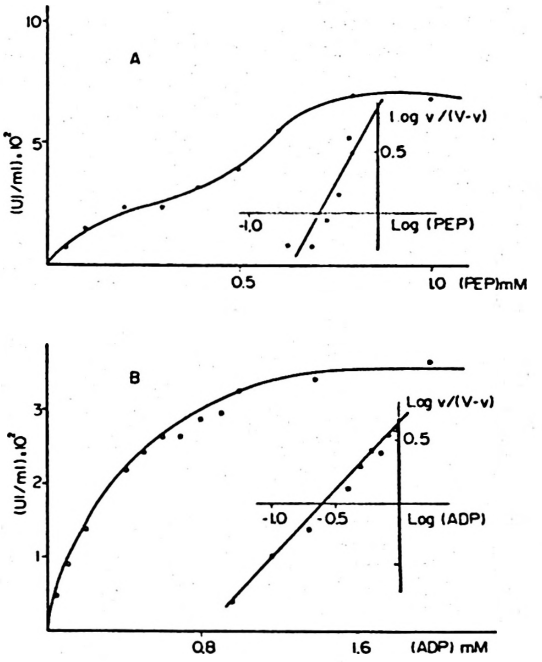

Fig. 1. *Activity variation of sea bass muscle PK versus both substrate concentrations. Hill plots are inserted.*
A. Substrate PEP. B. Substrate ADP.

This Feed-Forwarde activation of fish muscle PK by F-1.6-P_2, according to JOHNSTON (13), could give rise to a rapid generation of anaerobic energy, as a result of a great increase in the glycolytic flow. The capacity for the rapid generation of energy in this way could have an adaptation significance in relation to certain types of locomotor activity, such as swimming at a great velocity while escaping from predators. In salmonid white muscle more than 50 % of the muscle glycogen can be degraded after swimming at high speeds for two minutes.

Alanine and ATP are some of the most studied negative effectors on PK from different sources, with different behaviour according to the type of isozymatic PK. No effect was found at concentration of these compound from 0.5 to 10 mM. The concentration of PEP was 0.2 mM.

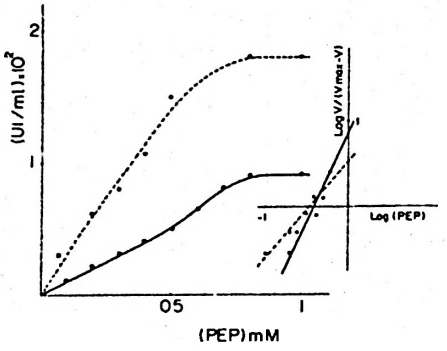

Fig. 2. *Effect of F-1.6-P_2 on sea bass muscle PK activity. Hill plots are inserted.*
(•) Without F-1.6-P_2. (o) + 0.01 mM F-1.6-P_2.

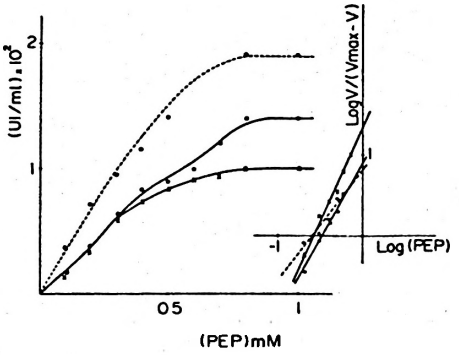

Fig. 3. *Effect of 1 mM phenylalanine on sea bass muscle PK activity. Hill plots are inserted.*
(•) Control. (×) plus 0.1 mM phenylalanine. (o) plus 0.1 mM phenylalanine + 0.01 mM F-1.6-P_2.

L-Phenylalanine (L-Phe) is also a clear inhibitor of PK. Diverse authors have reported this effect in fish tissue (13,20). In addition, the homologous enzyme from rat skeletal muscle is not regulated by alanine although it is inhibited by L-Phe.

There is a clear inhibitor effect on sea bass muscle PK (30 % inhibition at 2 mM PEP with 1 mM phenylalanine). Higher concentrations produced only a slight increase in inhibition, reaching a 50 % at a concentration of 8 mM L-Phe. The effect of 1 mM L-Phe is minor or insignificant when the concentration of PEP is closest to physiological values (fig. 3), characteristic of a competitive inhibition. The presence of L-Phe produces an increase in positive cooperativity with respect to PEP, and changes the $K_{0.5}$ value and Hill coefficient to 0.27 mM and 2.3 respectively. F-1.6-P_2 eliminates the inhibitor effect of L-Phe and displays its activator effect. F-1.6-P_2 does not modify the value of $K_{0.5}$ in the presence of L-Phe but decreases positive cooperativity (h = 1.3). Carbohydrates are the main energy source of mammals. Nevertheless in fish, proteins play a predominate role in the production of energy (23) and the requirements of essential amino acids are higher in these animals, approximately double that in rats and pig (18). The biosynthesis of non-essential amino acids, like the pyruvate group, in fish can be inhibited or regulated, by L-Phe for example, when there are sufficient proteins to meet the requirements of the cells. This inhibition by L-Phe is also a characteristic of muscle PK of mammals (11,12). In rats, the only amino acids that inhibit muscle PK is L-Phe, although at higher concentrations (5 mM). L-Phe is the regulatory amino acid that produces a marked effect on the PK M-type of rat liver, exhibiting an inhibition of 60 % with 1 mM L-Phe (12). The sensitivity of PK to L-Phe varies in different organisms. American eel muscle enzyme, *Anguilla rostrata*, shows only a 10 % inhibition in the presence of 4 mM L-Phe (20). On the contrary, for carp red skeletal muscle, *Carassius carassius* L., a 50 % inhibition of the enzyme was reached with 2 mM concentrations of L-Phe (13). The difference with respect to sea bass muscle PK, can be due to the fact that the assay was carried out at a less than 6.8 pH (closer to the physiological value), while the pH in our case was 7.4. Muscle PK of sea bass is not inhibited by ATP nor by alanine, contrary to what occurs in the liver of this species (5). Liver has the ability to carry

out gluconeogenesis and it, therefore, must have a considerably different set of metabolic controls from muscle.

In some circumstances, in which a higher energetic power is required, such as in rapid swimming, F-1.6-P_2 would activate sea bass muscle PK and eliminate the inhibition effect of phenylalanine as well, in which case a more activated glycolysis will increase pyruvate production and obtain energy via the Krebs cycle.

We can summarize these facts by saying that sea bass muscle PK is regulated by the following effectors: the substrate PEP, which presents positive cooperativity; F-1.6-P_2 which has an activator effect and L-Phe which acts as an inhibitor. These regulating properties are very similar to those shown by the isozyme M_2 from mammal (also called PK-K, PK-C or PK-4) and also very similar to those found for *A. rostrata* white muscle (20).

It must also be indicated that in sea bass muscle (*Dicentrarchus labrax* L.) no other isozymatic forms of PK have been found, since only one activity peak has been detected in the chromatograms and electrophoregrams.

Acknowledgements

The authors wish to express their gratitude to «Esperanza Siglo XIX» (Cádiz, Spain) for the gift of sea bass.

Resumen

La piruvatoquinasa de músculo de lubina presenta cooperatividad positiva respecto al sustrato PEP y es regulada por fructosa-1.6-bifosfato y L-fenilalamina. No es inhibida por alanina ni por ATP, aun cuando lo es por L-fenalanina. Los resultados de experimentos con extracto crudo, respecto al sustrato PEP, parecen indicar que la enzima se encuentra activada *in vivo* por la F-1.6-P_2. Se puede concluir que esta piruvatoquinasa presenta propiedades cinéticas y reguladoras similares a las mostradas por la isoenzima M_2 de mamífero.

Palabras clave: Lubina *(Dicentrarchus labrax* L.), Músculo, Piruvato quinasa.

References

1. Bannister, J.V. and Anastasi, A.: *Comp. Biochem. Physiol.*, 53 B, 449-451, 1976.
2. Bergmeyer, H.U. In «Methods of enzymatic analysis». (Bergmeyer, H.U. ed.) (2nd edn.) Academic Press. New York, 1974. Vol. 1. p. 510.
3. Black, J.A. and Henderson, M.H.: *Biochem. Biophys. Acta*, 284, 115-127, 1972.
4. Carbonell, J., Feliu, J.E., Marco, R. and Sols, A.: *Eur. J. Biochem.*, 37, 148-156, 1973.
5. Fideu, M.D., Pérez, M.L., Ruiz-Amil, M. and Herranz, M.J.: *Comp. Biochem. Physiol.*, 82 B, 841-848, 1985.
6. Fideu, M.D., Pérez, M.L., Herranz, M.J. and Ruiz-Amil, M.: *Comp. Biochem. Physiol.*, 80 B, 623-628, 1985.
7. Gaitán, S., Tejero, C. and Ruiz-Amil, M.: *Comp. Biochem. Physiol.*, 74 B, 801-805, 1983.
8. Guderley, H. and Cardenas, J.M.: *J. Exp. Zool.*, 211, 185-198, 1980.
9. Guderley, H. and Cárdenas, J.M.: *J. Exp. Zool.*, 212, 269-277, 1980.
10. Imamura, K. and Tanaka, T. In «Methods in Enzymology». (Wood, W.A. ed.). Academic Press. New York, 1982. Vol. 90. pp. 150-165.
11. Izbicka-Dimitrijevic, E., Mastalerz, F. and Kochman, M.: *Eur. J. Biochem.*, 114, 565-568, 1981.
12. Jiménez de Asua, E., Rozengurt, E., Devalle, J.J. and Carminatti, H.: *Biochim. Biophys. Acta*, 235, 326-334, 1971.
13. Johnston, I.A.: *Biochem. Biophys. Res. Commun.*, 63, 115-120, 1975.
14. Llorente, P., Marco, R. and Sols, A.: *Eur. J. Biochem.*, 13, 45-54, 1970.
15. Lowry, O.M., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: *J. Biol. Chem.*, 193, 265-275, 1951.
16. Munday, K.A., Giles, I.G. and Poat, P.C.: *Comp. Biochem. Physiol.*, 67, 403-411, 1980.
17. Mustafa, T., Moon, T.W. and Hochachka, P.W.: *Am. Zool.*, 11, 451-456, 1971.
18. Ogino, C., Chiou, J.Y. and Taneuchi, T.: *Bull. Jap. Soc. Sci. Fish.*, 42, 213-218, 1976.
19. Randall, R.F. and Anderson. P.J.: *Biochem. J.*, 145, 569-573, 1975.

20. Roberts, B. and Anderson, P.J.: *Comp. Biochem. Physiol.*, 80 B, 51-56, 1985.
21. Seubert, W. and Shoner, W. In «Current topics in cellular regulations». (Horecker, B.L. and Stadman, E.R. eds.) Academic Press. New York, 1971. Vol. 3. pp. 257-267.
22. Somero, G.N. and Hochachka, P.W.: *Biochem. J.*, 110, 395-400, 1968.
23. Waarde, A.V.: *Comp. Biochem. Physiol.*, 74 B, 675-684, 1983.
24. Walker, P.R. and Potter, V.R.: *J. Biol. Chem.*, 248, 4.610-4.616, 1973.
25. Yasumasu, I., Tazuwa, E. and Fujimara, A.: *Exp. Cell. Res.*, 93, 166-174, 1975.
26. Zammit, V.A., Beis, I. and Newsholme, E.A.: *Biochem. J.*, 174, 989-998, 1978.