REVISTA ESPAÑOLA DE FISIOLOGIA, 45 (2), 179-186, 1989

Regulation of Glycolysis in Sea Bass Liver: Phosphofructokinase Isozymes

M. D. Fideu*, M. L. Pérez, M. J. Herranz and M. Ruiz-Amil**

Departamento de Bioquímica
Facultad de Veterinaria
Universidad Complutense
28040 Madrid (Spain)

(Received on August 2, 1988)

M. D. FIDEU, M. L. PEREZ, M. J. HERRANZ and M. RUIZ-AMIL. *Regulation of Glycolysis in Sea Bass Liver: Phosphofructokinase Isozymes.* Rev. esp. Fisiol., 45 (2), 179-186, 1989.

Sea bass (*Dicentrarchus labrax* L.) liver phosphofructokinase (PFK) presents biphasic kinetics with respect to fructose-6-phosphate (F-6-P) in experiments carried out with crude extract. After the enzyme had been purified, two isozymes have been detected after chromatografic treatment. The two isozymes present different kinetic behaviour PFK-L_1, the first eluted phosphofructokinase activity shows positive cooperativity with respect to fructose-6-phosphate and PFK-L_2, the second activity fraction, has a Hill coefficient of 0.38 (negative cooperativity). The first isozyme shows less affinity for fructose-6-phosphate than that shown by PFK-L_2. The joint kinetics of both isozymes produces a biphasic kinetics with respect to fructose-6-phosphate, similar to that observed in crude extracts.

Key words: Sea bass, Liver, Isozymes PFK.

In the last several years aquaculture has demonstrated great interest in sea bass (*Dicentrarchus labrax* L.), because of its nutritional and gastronomic characteristics. Lipids and proteins seem to be the principal source of energy of fish (29), but under certain conditions fish are able to utilize carbohydrates for this purpose (22, 25) generally metabolized by pentose phosphate pathway (1, 2, 30). Moreover, key glycolytic enzymes can be induced due to a diet with a high content of carbohydrates, as it has been described on trout liver (9).

Carbohydrates are important in the study of energetic metabolism of sea bass. These studies will help to establish the nutritional requirements, determining the ranges of use of carbohydrates in the diet, as well as resulting in a lower production and maintenance cost.

PFK (ATP: D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11), a key regulatory enzyme of glycolysis, catalyses the phosphorilation of fructose-6-phos-

* To whom correspondence should be addressed.
** Present address: Departamento de Bioquímica. Facultad de Farmacia. Universidad Complutense. 28040 Madrid (Spain).

phate (F-6-P) in the presence of ATP and magnesium to yield fructose-1,6-bisphosphate (F-1,6-P_2) and ADP.

The properties of this enzyme from various animal sources have been investigated, and multiple molecular forms have been detected in several species (8, 23). Four chromatographically different PFK isozymes from rat were found: PFK-I in the muscle, heart and brain; PFK-IV in liver and erythrocytes; and PFK-II and PFK-III forms in the brain, spleen, kidney and testes; the latter has also been detected in stomach.

The denomination of these isozymes corresponds to the elution position in ion-exchange chromatography (DEAE cellulose). Studies on the immunological properties of these enzymes seem to indicate that at least three enzymes exist in different rat tissues (8). On the other hand, DUNAWAY and WEBER (5) and DUNAWAY *et al.* (6, 7), by means of chromatographic and electrophoretic techniques, have reported three enzymes in rat tissue: PFK-M (muscle), PFK-L_1 (minor liver PFK isozyme) and PFK-L_2 (the major hepatic PFK isozyme).

To date, no detailed study has been carried out on PFK purified from fish livers. Only SAND (20) has notified the existence of two isozymes with the same molecular weight but differing in charge distribution of phosphofructokinase from liver of the flounder (*Platichtys flesus* L.).

In this research sea bass liver PFK has been purified and its kinetic behaviour studied. A purification method, which allows the separation of the two isozymes in only one step is described. We have studied the possible infuence of the most characteristic effectors of the enzyme in crude extracts and in the two isozymes.

Materials and Methods

Biological Material. — Sea bass (*Dicentrarchus labrax* L.) were obtained from «Salina Esperanza Siglo XIX» (Cádiz, Spain) where they were maintained with controlled diet and optimal oxigenation and salinity conditions. The temperature varied with the season (10-28° C).

Enzyme assays. — Phosphofructokinase activity was estimated according to Layzer (16) by measuring the rate of NADH oxidation in a coupled system with aldolase, triosephosphate isomerase and phosphoglycerol dehydrogenase. In all experiments the lack of existence of NADH oxidation has been proven, initiating the reaction on adding F-6-P. In a final volume of 1 ml, the reaction contained: Tris-HCl, pH 8, 100 µmol; KCl, 94.5 µmol; $MgCl_2$, 3.6 µmol; F-6-P, 1 µmol; G-6-P, 3 µmol; ATP, 3.5 µmol; aldolase 1.8 IU; phosphoglycerol dehydrogenase 2 UI; triosephosphate isomerase 6 IU and 25 µl of enzyme extract (containing 2 mg of protein). Enzyme units are expressed as micromoles of substrate utilized or product formed per minute. All experiments were carried out at 22° C.

Protein determination. — Protein was determined by the methods of LOWRY *et al.* (18).

Purification of the enzyme. — The purification steps were carried out according to the following procedure and are summarized in table I. All the experiments were performed at 4° C. To obtain the crude extracts, the liver (4 g) was homogenized with 4 ml of 50 mM phosphate buffer pH 8. The suspension was centrifuged at 26,000 × *g* for 30 minutes. The supernatant layers were applied to a 17 × 1 cm column of Blue Sepharose CL-6B previously equilibrated with 50 mM phosphate buffer pH 8 containing 0.2 mM fructose-6-phosphate and 5 mM 2-mercaptoethanol (MET).

Once the extract was added, 50 mM monopotassic phosphate, pH 8, containing

5 mM MET was used as wash buffer. Then the same wash buffer with added 9 mM ADP was used for the elution. The low rate was 15 ml/h at constant pressure, 2 ml fractions were collected.

Electrophoresis. — The polyacrylamide gel electrophoresis was carried out according to the method described by DAVIS (4). The intensity used was 213 mA per tube and the tris-glycine buffer had pH 8.3.

Determination of the molecular weight. — A 38 × 2 cm. Sephacryl S-300 column equilibrated with 65 mM Tris-HCl pH 7.5, containing glycerol was used. Samples of the crude extract were never superior to 0.5 mg protein/ml of the gel. Elution buffer was the same as described above. The flow was 15 ml/h at a constant pressure. Two ml fractions were collected and the phosphofructokinase activity was determined. The standard curve was obtained using a mixture of the following globular proteins: 10 mg chicken egg albumine (67,000), 6 mg rabbit muscle aldolase (158,000), 8.5 mg bovine liver catalase (232,000), 1.9 mg horse spleen ferritin (440,000) and 10 mg bovine thyroid thyroglobuline (669,000) at a final volume of 1.5 ml. The equilibration and elution of the column was performed under similar conditions as previously described.

Chemical and reagents. — Nicotinamide adenine dinucleotide reduced form (NADH), fructose-6-phosphate (F-6-P), glucose-6-phosphate (G-6-P), adenosine-5-triphosphate (ATP) were purchased from Boehringer-Manhein. Other reagents of analytical grade were purchased from Merck. Chromatographic material (columns, collector, Uvicord, etc.) was purchased from LKB. Sephacryl S-300 and Blue-Sepharose CL-6B were from Pharmacia.

Results and Discussion

Experiments carried out with crude extracts have demonstrated that PFK of sea bass liver has a biphasic behaviour with respect to F-6-P (fig. 1, graph. B) (10). During the first phase of the curve (at small concentrations of substrate) PFK presents negative cooperativity, whereas in the second phase of the curve (at high concentrations of the substrate) it presents positive cooperativity. One of the three possible explanations that we have suggested for this type of kinetics, was the presence of isozymes with different affinity for the substrate. Nevertheless, results reported at that moment seemed to be insufficient since the activity curve with respect to the pH, displayed only one max-

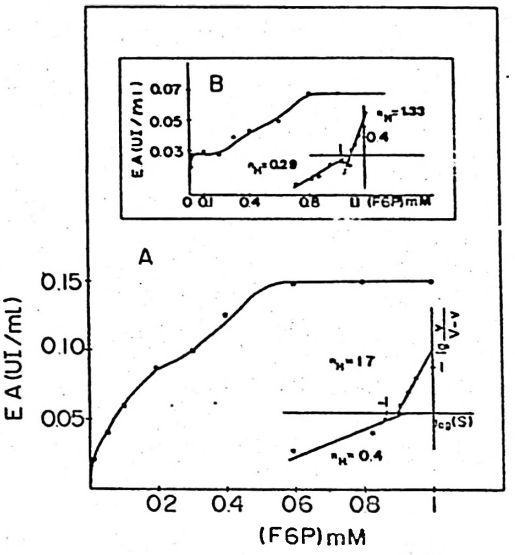

Fig. 1. *Phosphofructokinase isozymes in sea bass liver.*

A: Phosphofructokinase activity sum of PFK-L_1 and PFK-L_2 activities with respect to the F-6-P concentration. B: Phosphofructokinase activity vs. F-6-P in crude extracts. Hill plots are inserted. The values represented are average values of at least three experiments. In both cases, a temperature of 22° C and constant concentration of 3.5 mM Mg-ATP was maintained.

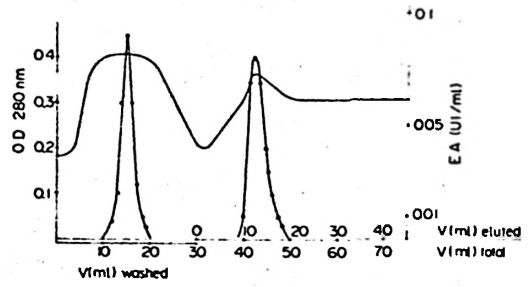

Fig. 2. *Blue Sepharose CL 6B chromatography of sea bass liver PFK.*
Phosphate buffer (pH 8) was used, containing F-6-P (0.2 mM) for equilibrated and ADP (9 mM) for elution.

imum at pH 8 and not two as could be expected. To test the previous hypothesis a purification process was carried out.

Purification processes are often tedious and the recovery is low. We have been able to purify PFK from sea bass liver using only one step with Blue Sepharose CL-6B, affinity chromatography with a 100 % recovery. Crude extracts were used since temperature denaturalization displayed by this enzyme does not permit a thermic treatment prior to the chromatographic process. The results obtained are shown in fig. 2, two peaks of phosphofructokinase activity were observed. The first enzymatic activity elutes with wash buffer solution and is called PFK-L_1, the second called PFK-L_2, elutes when ADP (9 mM) is added to the previous dissolution buffer. The level of purification obtained is 75 and 266 respectively with a recovery of 50 % for each enzymatic activity (table I). These results seem to indicate the possible existence of two isozymes of sea bass liver PFK.

The electrophoretic development with polyacrylamide gel exhibits only one band of protein using the eluted fractions with activity corresponding to the PFK-L_2. When wash fractions (PFK-L_1) are used, a number of bands appear, but none of them have the same R_f value as that corresponding to PFK-L_2. These facts seem to confirm the existence of two isozymes of sea bass liver PFK.

The molecular weight of both isozymes seem to be very similar since the separation with Sephacryl S-300 chromatography was not possible. This process allow the calculation of the molecular weight, being 400,000 ± 37,000. Experiments carried out with Sephacryl S-200 using PFK-L_1 fraction support this theory. These data are in agreement with those found by SAND (20) for flounder-liver (*Platichtys flesus* L.) in the sense that this author reported the existence of two isozymatic forms with the same molecular weight but differing in charge distribution.

On the other hand, none of the fractions with PFK activity obtained, by affinity chromatography exhibited a biphasic kinetic behaviour with respect to F-6-P (fig. 3). PFK-L_1 displays a slightly sigmoidal behaviour with F-6-P; Hill coefficient and $S_{0.5}$ values are 1.3 and 0.22 mM respectively. The kinetic behaviour of PFK-L_2 is different, its Hill coefficient is 0.38 (negative cooperativity) and $S_{0.5}$ is

Table I. *Summary of the purification of sea bass liver phosphofructokinase.*

		Activity (UI · ml^{-1})	Proteins (mg · ml^{-1})	Specific activity (UI · mg^{-1})	Purification Factor	Yield %
Homogenate		0.36	58.5	0.006	—	100
Blue Sepharose	PFK-L_1	0.09	0.20	0.45	75	50
	PFK-L_2	0.08	0.05	1.60	266	50

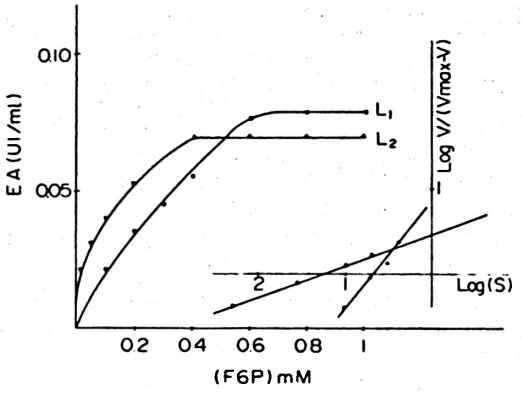

Fig. 3. *Fructose 6 phosphate effect on PFK-L_1 (•) and PFK-L_2 (▲) activities.*
Hill plots are inserted. Mg-ATP constant concentration: 3.5 mM. PFK-L_1 (•): $S_{0.5}$ = 0.22 mM, n_H = 1.3. PFK-L_2 (▲): $S_{0.5}$ = 0.05 mM, n_H = 0.38.

0.05 mM. This isozyme is saturated at low concentrations of F-6-P (0.4 mM). These results coincide with those of the crude extract. The superior part of the biphasic curve of the crude extract (fig. 1, graph. B) can correspond with a predominant PFK-L_1 phosphofructokinase activity and the inferior part with PFK-L_2, since they act simultaneously. This hypothesis is supported by the fact that a biphasic curve is obtained when the results for the two purified fractions are collected (fig. 1, graph. A). This is more evident in the Hill representation where $S_{0.5}$ and n_H values are similar to those obtained in crude extract (fig. 1, graph. B).

These results at 22° C are also important since they imply a higher validity to thermoregulation mechanisms of sea bass liver phosphofructokinase activity proposed by us and based on the changes on $S_{0.5}$ and n_H observed in crude extracts for each part of the biphasic curve (10). These mechanisms can permit a constant glycolytic activity, independent of the temperature, in a range that can be considered more normal in an artificial habitat (during culture in zones with a Mediterranean climate). Based on the data reported by FIDEU *et al.* (10) it is possible to indicate that the isozyme PFK-L_2 (with higher affinity for the substrate, and that acts at lower concentration of the substrate) is the most operative to reach an independent phosphofructokinase activity with respect to temperature.

On the other hand, the existence of numerous effectors that regulate this enzymatic activity in different organisms has been described. PFK is a multi-modulated enzyme prototype: We have studied the possible effect of fructose-2,6-bis-phosphate (F-2,6-P_2); adenosine-5'-monophosphate (AMP); adenosine-3':5'-monophosphoric acid cyclic (c-AMP); adenosine-5'-diphosphate (ADP); inorganic phosphate (Pi); phosphoenolpyruvate (PEP) and citrate as the most characteristic modulators of this activity (12, 14, 17, 19, 21, 27, 28) in crude extracts of PFK as well as in the PFK-L_1 and PFK-L_2 isozymes, with the aim of determining the possible differences in the regulation of these enzymatic activities. The chosen concentrations of these effectors are within the physiological range described for fish (12).

The results of the experiments carried out in crude extracts are shown in table II, where the examined concentrations are included for those in which modulation has been observed. Cyclic-AMP (0.05, 0.1 mM), citrate (0.1, 0.2, 0.6 mM) and Pi (10, 20 mM) have displayed no effect. F-2,6-P_2 and PEP act as activators of sea bass liver PFK.

It is interesting to note that the intensity of the influence shown by these compounds differs in the two sections of the biphasic curve that this enzyme presents in crude extracts with respect to the substrate fructose-6-phosphate. In this sense F-2,6-P_2 (0.1 mM) exhibits an activating effect in the lower section (0.01 mM F-6-P), an effect not seen in the higher section (0.4 mM F-6-P).

PEP behaves as an activator, unlike

Table II. *Influence of different effectors on sea bass liver PFK activity (%).*

Effectors / concentration (mM)	Crude extract		PFK-L_1	PFK-L_2	
	F-6-P (0.4 mM)	F-6-P (0.01 mM)	F-6-P (0.4 mM)	F-6-P (0.4 mM)	F-6-P (0.01 mM)
F-2,6-P_2; 0.1	No effect	(+) 10	No effect	—	—
PEP 1.0; 2.0	(+) 100	(+) 250	(+) 500	—	—
0.1	No effect	No effect	—	—	—
ADP 1.0	No effect	No effect	—	—	—
9.0	(−) 25	(−) 50	(−) 50	—	—
ADP (9.0) PEP (1.0)	(+) 85	(+) 200	(+) 600	(+) 900	(+) 1600
ADP (9.0) F-2,6-P_2 (0.1)	—	(−) 45	—	No effect	(+) 30

(+) Activator effect. (−) Inhibitor effect.

what is observed in this same enzyme from other organisms (3, 15, 17, 26, 27). This activation is more intense in the lower part of the saturation curve with respect to fructose-6-phosphate, the same as in the case of the previously mentioned effector.

ADP as reported by different authors, shows a different behaviour on the PFK activity of different species (8, 17, 21, 24, 27). This nucleotide did not show inhibition of sea bass liver PFK at physiological concentration (0.1, 1 mM) but ADP at 9 mM behaves as an inhibitor, and its effect is also more intense at concentrations of F-6-P inferior to 0.1 mM.

The activities of the crude extract and PFK-L_1 were assayed in the presence of 9 mM ADP, in order to be compared with PFK-L_2, which is affected by this nucleotide at this concentration, after being eluted in the affinity chromatography (see Materials and Methods).

All these results demonstrate a greater effect of these compounds when the concentration of F-6-P is closer to physiological levels. Moreover, the inhibitor effect observed with ADP is annulled in the presence of PEP.

The compounds that showed regulation of this phophofructokinase activity in the crude extract were those that were tested in samples of isozymes PFK-L_1 and PFK-L_2 (table II). The concentration of substrate fructose-6-phosphate used were 0.4 mM for this study with PFK-L_1 and 0.01 mM for the PFK-L_2 isozymes, concentrations at which the experiments with crude extract have been carried out and clearly correspond to the two different sections of the biphasic saturation curve. In the case of PFK-L_2, 0.4 mM of F-6-P was also used to compare the behaviour of the different effectors on both isozymatic forms at the same substrate concentration.

Qualitatively, the effect of these compounds on both isozymes is similar to that observed when the same fructose-6-phosphate concentrations are used in crude extract. Quantitatively, this effect is somewhat different (table II). Since eluted PFK-L_2 fractions contained ADP from the elution medium, the isolated effect of these compound was not investigated for this isoenzyme.

The maximun effect of all these modulators can be observed in concentration 0.01 mM F-6-P and PFK-L_2 isozymatic species, the results for 0.4 mM F-6-P are very similar for both isozymatic species. All these results seem to indicate differences in the regulation of both isozymatic forms.

The slight or non-existent influence found in some significant modulators of the PFK of other origins, such as F-2,6-

P_2 or AMP and citrate could be due to a slight inhibition (11) produced by 3.5 mM Mg-ATP used in the experiments. The existence of a positive modulation of the glycolysis in sea bass liver by PEP, probably necessary when there is a reduction of lipids and aminoacids, can be shown. A similar activator effect of PEP has been observed in PFK of trout haematopoietic cells, which is not inhibited by ATP (13). The metabolism of carbohydrates in fish is fundamentally performed by the pentoses phosphate pathway. The production of PEP will activate glycolysis, which can indicate that both routes are operative in livers of this aquatic species, at the indicated physiological conditions.

It is concluded that the activity phosphofructokinase of sea bass liver is regulated by two isozymes. One (PFK-L_2) can be isolated pure by chromatographic treatment with Blue Sepharose CL-6B, presents negative cooperativity and has high affinity for the substrate F-6-P. This enzyme has greater sensitivity to the effect of the modulators and to F-6-P concentrations, which can be considered in the physiological range (12).

Acknowledgements

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

Resumen

La fosfofructoquinasa (PFK) de hígado de lubina (*Dicentrarchus labrax L.*), presenta cinética bifásica con respecto a la fructosa-6-fosfato (F-6-P) en experimentos realizados con extracto crudo. Debido a esto, la enzima se purifica mediante tratamiento cromatográfico, detectándose dos isoenzimas que manifiestan distinto comportamiento cinético. PFK-L_1, la primera en eluir, muestra cooperatividad positiva respecto a la F-6-P. PFK-L_2, por el contrario, presenta un coeficiente de Hill de 0,38 (cooperatividad negativa). La primera isoenzima manifiesta, además menor afinidad por la F-6-P que la PFK-L_2. La cinética conjunta de ambas isoenzimas produce una cinética bifásica respecto al sustrato F-6-P, similar a la observada con extracto crudo.

Palabras clave: Lubina, Hígado, Isoenzimas de PFK.

References

1. Baldwin, J. and Reed, K. C.: *Comp. Biochem. Physiol.*, 54 B, 527-529, 1976.
2. Bautista, J. M., Garrido-Pertierra, A. and Ruiz-Amil, M.: *Comp. Biochem. Physiol.*, 77 B, 843-848, 1984.
3. Bosca, L., Aragón, J. L. and Sols, S.: *Biochem. Biophys. Res. Commun.*, 106, 486-491, 1982.
4. Davis, B. J.: *Ann. N.Y. Acad. Sci.*, 121, 404-427, 1964.
5. Dunaway, G. A. and Weber, G.: *Arch. Biochem. Biophys.*, 162, 620-628, 1974.
6. Dunaway, G. A., Morris, H. P. and Weber, G.: *Cancer. Res.*, 34, 2209-2216, 1974.
7. Dunaway, G. A., Leung, G. L. Y., Cooper, M. D., Thrasher, J. R. and Wagle, S. R.: *Biochem. Biophys. Res. Commun.*, 80, 71-74, 1978.
8. Dunaway, G. A.: *Moll. Cell. Biochem.*, 52, 75-91, 1983.
9. Fideu, M. D., Soler, G., Ruiz-Amil, M.: *Comp. Biochem. Physiol.*, 74 B, 795-799, 1983.
10. Fideu, M. D., Pérez, M. L., Herranz, M. J. and Ruiz-Amil, M.: *Comp. Biochem. Physiol.*, 80 B, 623-628, 1985.
11. Fideu, M. D., Herranz, M. J., Ruiz-Amil, M. and Pérez, M. L.: *Comp. Biochem. Physiol.*, 81 B, 1067-1071, 1985.
12. Freed, J. M.: *Comp. Biochem. Physiol.*, 39 B, 747-764, 1971.
13. Gaitan, S.: Tesis Doctoral. Complutense Universidad. Madrid, Spain, 1987.
14. Jensen, G. S.: *Comp. Biochem. Physiol.*, 70 B, 161-164, 1981.
15. Kretschmer, M. and Hofman, E.: *Biochem. Biophys. Res. Commun.*, 124, 793-796, 1984.
16. Layzer, R. B.: In «Methods in Enzymology» (Wood, W. A., ed.), Academic Press, New York, 1975, pp. 109-110.
17. Leavert, I. and Burt, J. R.: *Comp. Biochem. Physiol.*, 69 B, 127-132, 1981.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J.: *J. Biol. Chem.*, 193, 265-275, 1951.
19. Nissler, K., Otto, A., Schellenberg, W. and

Hofman, E.: *Biochem. Biophys. Res. Commun.*, 111, 294-300, 1983.
20. Sand, O.: *Comp. Biochem. Physiol.*, 68 B, 77-81, 1981.
21. Sand, O.: *Comp. Biochem. Physiol.*, 69 B, 435-443, 1981.
22. Smith, R. R.: *Prog. Fish. Cult.*, 33, 132-134, 1971.
23. Tanaka, T., An, T. and Sakue, Y.: *J. Biochem.*, 69, 609-612, 1971.
24. Tanaka, T., Imamura, K., An, T. and Taniuchi, K.: *Gann. Monogr. Cancer. Res.*, 13, 219-234, 1972.
25. Ushiyama, H., Fujimara, T., Shibata, T. and Yoshimara, K.: *Hokkaido Daigaku Suisan Yakubu Kenkyu Iho*, 16, 183-188, 1965.
26. Uyeda, K. and Racker, E.: *J. Biol. Chem.*, 240, 4632-4688, 1965.
27. Uyeda, K.: In «Advances in enzymology» (Meister, A., ed.). Academic Press, New York, 1979, pp. 193-244.
28. Van Schaftingen, E., Jett, M. F., Hue, L. and Hers, H. G.: *Proc. Natl. Acad. Sci. USA*, 78, 3483-3486, 1981.
29. Waarde, A. V.: *Comp. Biochem. Physiol.*, 74 B, 675-684, 1983.
30. Yamauchi, T., Stegeman, J. J. and Goldberg, E.: *Arch. Biochem. Biophys.*, 167, 13-20, 1975.