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Regulation of the Pentose Phosphate Cycle in Bass (*Dicentrarchus labrax* L.) Liver

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The influence in the activities of the glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase produced by the ratio changes NADPH/NADP is studied. The intracellular ratios of 20 and 10 are enough to achieve total inhibition of these two enzymes, respectively. Measurements of a number of metabolic intermediates show that the concentrations of Krebs cycle compounds are higher than those of glycolytic pathway metabolites. From a consideration of these values, the regulation of the pentose phosphate cycle mainly by the intracellular NADPH/NADP ratio, is discussed.

Key words: Pentose phosphate cycle, Regulation, NADPH/NADP.

The central pathways of intermediary metabolism which have been studied in salmonids are very similar to those occurring in other species. Nevertheless, some differences seem to exist due to diet and environmental conditions. So that the energy requirements of these ectothermic animals are lower than those of mammals which expend much more energy to maintain their body temperature.

Some authours (2, 11, 12, 21, 22) suggest that diet carbohydrates are poorly utilized as an energy souce and that most energy proceeds from protein catabolism. Aminoacids are also the main substrates of gluconeogenic pathways. These and other anabolic pathways require ATP and NADPH which are produced mainly in the respiratory chain and in the pentose phosphate cycle, respectively.

Although many of the enzymes of the pentose phosphate cycle have been found in fish tissues, the precise physiological significance and its control remain unclear. An apparent unbalance between the activities of 6PGDH* and G6PDH, enhanced by the fact that NADPH is a more powerful competitive inhibitor of the

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Abbreviations used: G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; F-1,6-PP, fructose-1,6-diphosphate.

6PGDH enzyme has been described by SAPAG-HAGAR *et al.* (25). At present in mammals, the fine control of the pentose phosphate pathway is being focused on NADPH-producing enzymes by the effect of NADPH/NADP ratio (8). In previous papers (3, 13, 18), some properties of the purified NADPH-producing dehydrogenases from bass liver have been described, while in this paper, a study of the changes in the activities of the purified G6PDH and 6PGDH caused by the effect of NADPH is presented. These observations can contribute to a better understanding of the control of the pentose phosphate cycle in these animals.

Materials and Methods

Biological material. — The bass *Dicentrarchus labrax* L. from North Spain were dissected at 4°C and the livers were used fresh or frozen.

Enzyme assays. — To determine the G6PDH activity, the standard assay mixture contained in one ml: 0.1 M Tris-HCl buffer pH 7.2, 5 mM $MgCl_2$, 1 mM glucose-6-phosphate and 0.5 mM NADP (3). The activity of the 6PGDH was determined in the standard assay mixture containing in one ml: 50 mM Tris-HCl buffer pH 7.5, 5 mM $MgCl_2$, 2 mM 6-phosphogluconate and 0.5 mM NADP. Both reactions were started by adding 2 µg of purified enzyme proteins and followed at 340 nm using a Pye-Unicam SP8-100 spectrophotometer. One unit was defined as the amount of the enzyme catalyzing the reduction of 1 µmol/min of NADP at 30°C.

Preparation of the crude extracts. — To obtain the crude extracts, the liver was homogenized with a volume of 20 mM Tris-HCl pH 7.4 containing 9 % glycerol and 0.5 mM EDTA, three times its weight. The supernatant resulting from centrifugation at 13,000 *g* × 30 min at 4°C was used immediately or stored overnight in a frozen state.

Purification of the enzymes. — G6PDH and 6PGDH were purified until homogeneity using procedures of gel filtration, ion exchange and affinity chromatographies as previously described (3, 19).

Estimation of NADP and NADPH concentration. — The concentrations of NADP and NADPH were essentially estimated as described by GREENBAUM *et al.* (10). Either 0.2 ml 2N KOH for NADPH determinations, or 0.2 ml 20 % $KClO_4$, in the case of NADP determinations were added to 1 ml of crude extract. Both extracts were incubated at 60°C for 10 minutes and neutralized, and the soluble supernatants were used for NADP or NADPH estimation. The reaction mixture was held in the reaction chamber of a Clark-type electrode and contained 0.1 M Tris-HCl pH 8, 1.2 mM glucose-6-phosphate, 0.8 phenazine methasulphate, 6 mM EDTA, 0.5 µg of purified G6PDH, and different amounts of acid or alkaline extracts in a volume of 2 ml. The concentration of both nucleotides was estimated through the rate of oxygen consumption in the mixture, following the addition of G6PDH, using a Gilson oxygraph mod. K-1C. An internal standard of NADP or NADPH was carried out for each assay.

Estimation of metabolic intermediates. — One ml of the crude extract was suspended in 1.5 ml of 1.2 M $HClO_4$ at 0°C according to the method of LONG (16). The suspensions were shaken in a mixer and placed on ice for 15 minutes at 2°C. The supernatants were then neutralized with 1.08 M KOH containing 0.24 M $KHCO_3$. The $KClO_4$ precipitate was removed by centrifugation at 30,000 *g* at 4°C and the supernatants were stored on ice prior to analysis. Most of metabolic intermediates were determined by coupled

enzymatic assays (5), citrate was estimated by the method of MOELLERING and GRUBER (20).

Estimation of the redox state. — The NADPH/NADP and NADH/NAD ratios in the cytoplasm were calculated from the equations proposed by KREBS and VEECH (15).

Chemicals. — NAD, NADP, NADH, NADPH and glucose-6-phosphate were obtained from Sigma. All the other chemicals were of the highest purity commercially available.

Results

Table I shows the effect of NADPH/NADP ratio on the G6PDH and 6PGDH activities. As can be observed from data, both enzymes are almost totally inhibited by NADPH at the highest concentration assayed (1 mM), and values of this nucleotide ratio of 20 and 10 are enough to achieve total NADPH inhibition for G6PDH and 6PGDH, respectively. In the first case, the value is similar to the data reported by EGGLESTON and KREBS (7), RODRÍGUEZ-SEGADE *et al.* (24) and SILVA-PANDO *et al.* (27), who indicate ratio values between 9 and 20 to obtain similar inhibition of the enzyme. The affinity of the 6PGDH for NADP is six-fold that of G6PDH (table II) although the Ki/Km ratio value is 0.8 for both dehydrogenases. From these observations it is more likely that the adjustment of the activities of the two enzymes is achieved by control through deinhibition of them. Insufficient information of the properties of these dehydrogenases of the pentose phosphate cycle is available for a detailed analysis of the mechanism and kinetics of the NADPH effect. It should be of interest that G6PDH of bass liver occurs as a dimer and monomer, and that the dimer-monomer interconversion depends on the presence of NADP (4).

Table I. *Effect of NADPH/NADP ratio on velocity of the reactions catalyzed by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase from bass liver.*

[NADP] nM	Without NADPH	[NADPH] 0.06 mM	Inhibition (%)[b]	[NADPH] 0.20 mM	Inhibition (%)[b]	[NADPH] 1 mM	Inhibition (%)[b]
			GLUCOSE-6-PHOSPHATE DEHYDROGENASE				
0.01	0.06[a]	0.05[a]	21	0.01[a]	80	0	99
0.02	0.1	0.08	19	0.03	70	0	99
0.05	0.14	0.13	11	0.06	57	0.01[a]	96
0.01	0.17	0.16	7	0.09	48	0.01	90
0.25	0.19	0.18	4	0.13	30	0.03	85
0.5	0.19	0.19	2	0.14	28	0.05	76
			6-PHOSPHOGLUCONATE DEHYDROGENASE				
0.01	0.07[a]	0.05[a]	23	0.03[a]	83	0	100
0.02	0.1	0.08	21	0.03	72	0	100
0.05	0.15	0.13	13	0.06	60	0	99
0.1	0.17	0.16	7	0.08	53	0	96
0.25	0.19	0.18	4	0.11	40	0.01[a]	93
0.5	0.2	0.19	4	0.14	31	0.03	83

[a] The velocity is expressed in µmol NADP red./min.

[b] The % is calculated: $\%\ \text{Inhibition} = \left(1 - \frac{\text{Initial velocity with inhibitor}}{\text{Initial velocity without inhibitor}}\right) \times 100.$

Table II. *Kinetic and inhibition constants of producing-NADPH enzymes.*
Km and Ki values (μm) were determined for purified glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Km for NADPH-isocitrate dehydrogenase was determined in crude extracts. The standard isocitrate dehydrogenase assay consisted of 0.1 M Tris-HCl pH 7.2, 0.05 mM $McCl_2$, 0.1mM NADP and 1 mM isocitrate. For other standard assays see text.

Enzyme	Km NADP	Ki NADPH
Glucose-6-phosphate dehydrogenase	12	9.8
6-Phosphogluconate dehydrogenase	2	1.6
Malic enzyme	22	68.5
NADPH-Isocitrate dehydrogenase	25	—

Table III. *Intracellular concentrations of some metabolic intermediates in bass liver cytoplasm.*
Extracts were used from stop-frozen liver. The results are expressed as mean values ± SD for twenty six determinations.

Metabolite	Amount of metabolite (nmoles/g fresh liver)
Glucose-6-phosphate	107 (± 17)
Fructose-6-phosphate	26 (± 7)
6-Phosphogluconate	7 (± 3)
Dihydroxiacetone phosphate	20 (± 6)
Fructose-1,6-diphosphate	6 (± 3)
Phosphoenolpyruvate	32 (± 8)
Pyruvate	31 (± 8)
Lactate	461 (± 51)
Citrate	483 (± 89)
Aspartate	1,283 (± 123)
Malate	490 (± 26)
Glutamate	3,012 (± 284)
γ-Oxoglutarate	910 (± 117)
Acetoacetate	99 (± 10)
Isocitrate	440 (± 68)
NADP	212 (± 21)
NADPH	1,823 (± 137)

The intracellular concentrations of some metabolites in bass liver extracts are shown in table III. Some of the differences between these values are as expected. Thus, taking into account that protein is the main carbon source in these animals, the concentration of Krebs cycle intermediates is higher than the glucolytic compounds. The measurements of the mass-action ratio of the reaction catalysed by the glucose-6-phosphate isomerase enzyme are approximately equal to the equilibrium constant, which suggests that this reaction is close to the equilibrium. A striking feature revealed by table III, is the low values found for the 6-phosphogluconate and the F-1,6-PP. In the first case, it may suggest that the reaction catalysed by 6PGDH is displaced to the formation of ribose-5-phosphate and CO_2. The low value obtained for the F-1,6-PP may be indicative of the rather low activity of glycolysis, mainly because the pyruvate kinase in bass liver needs to be activated by that compound (9). In tissues such as liver and kidney and in many bacterial cells, the processes of glycolysis and gluconeogenesis occur in the same cell. When bacteria are grown on gluconeogenic compounds as a carbon source, the F-1,6-PP concentration is of the same order as the value found in bass liver by us, and this value increases some-fold when bacteria are grown on glucose or fructose (17).

Most of the metabolite concentrations (table III) are less than those obtained in well-fed rat liver by different authours (32), and similar to those found in rats that have been fed with low carbohydrate diets (15). This supports the fact that bass poorly utilize these compounds. Taking into account the malate, pyruvate, α-oxoglutarate and isocitrate concentrations (table III), as well as the correspondent equilibrium constants of the malate enzyme and NADP-linked isocitrate dehydrogenase reactions (15), the values of the NADPH/NADP ratio in the cytoplasm are 462 and 380. These values are six-fold and four-fold those described for well-fed rats. Likewise, taking into account the pyruvate and lactate concentrations and the equilib-

rium constant of the lactate dehydrogenase reaction, the NADH/NAD ratio is 2×10^{-3}. This value is similar to that described for rats fed with carbohydrate diets, and 2.5-fold that for well-fed rats (15).

Discussion

A characteristic property of the 6PGDH and G6PDH enzymes is the high influence of the NADPH inhibition. The Ki values obtained for these two dehydrogenases are smaller than those obtained for homologous enzymes from liver of different species (23, 25, 28, 30). Despite the fact that the intracellular concentrations of glucose-6-phosphate and 6-phosphogluconate are higher than their respective Km's, and that the control can be achieved by substrate limitations, the values of physiological ratio NADPH/NADP suggest that the short term regulation can be determined for the value of this ratio.

Most calculations of the cellular free NADPH/free NADP ratio have been based on the equilibrium constant and mass-action ratio of the decarboxylating enzymes. If this hypothesis is considered, the intracellular NADPH/NADP ratios are 462 and 380, when they are determined through the reaction catalysed by the malic enzyme and the NADP-linked isocitrate dehydrogenase (15). The Ki values found for these enzymes by us are higher than those obtained for homologous enzymes by ANDRES *et al.* (1). In these conditions, the activities of the dehydrogenases of the pentose phosphate pathway must be totally inhibited since ratio values as low as 20 and 10 are capable of inhibiting more than 95 % of the G6PDH and 6PGDH activities, respectively. However, the use of decarboxilating enzymes requires mainly that they have enough specific activity to act close to equilibrium as there is a physiological tendency to produce CO_2 since these enzymes use HCO_3 as substrate (6, 31). The CO_2 produced in metabolism is eliminated through enzymatic hydration (14). In agreement with these considerations, the use of the decarboxilases must produce an overestimation of the calculated NADPH/NADP ratio, and the values quoted should also be high.

If, as it is suggested by SOLS and MARCO (29), the intracellular NADPH/NADP ratio is closer to that measured as total than to that calculated as free (26), the value of this ratio is 8.6 in bass liver (Table III). This is close to those rates which permit the dehydrogenases of the pentose phosphate cycle to be operative, as small variations in the NADPH/NADP ratio in this order of magnitude could effectively modulate the cycle flux. The variation of the NADPH/NADP ratio could depend on the relation between activities of NADPH-producing pathways and NADPH-consuming pathways. In rat tissues, the activity of the NADPH-producing pathways is several-fold that of the consuming-pathways (8). But this situation should be different in bass, where proteins seem to be the main carbon source. Furthermore the reduction of NADP-concomitant to glucose-6-phosphate and/or 6-phosphogluconate oxidation must play a crucial role in the provision of reducing equivalents with biosynthetic purposes, while the control of these enzymes by changes of NADPH/NADP ratio seems to have an important physiological significance.

Some authors have proposed the existence of cellular factors capable of overcoming the NADPH inhibition. In rat liver, EGGLESTON and KREBS (7) reported that the oxidized glutathione (GSSG) in combination with an unidentified cofactor, are responsable for that effect. RODRÍGUEZ-SEGADE *et al.* (24) described the presence of a small protein in mussel hepatopancreas, which reversed the NADPH inhibition. Finding a protein similar to that in bass liver extracts, by

following the same procedure as described by these authours, was unsuccessfully attempted by us. Nevertheless, it has been found that small changes in pH can influence the inhibition of the G6PDH enzyme by the nucleotide (3). The absence of factors which reverse the NADPH inhibition does not seem surprising, since the variations due to nutritional and environmental factors, can influence the liver enzyme activities in these ectothermic animals. Such an influence may be sufficient to cause this effect, promoting a rapid adjustment on 6PGDH and G6PDH activities and on their inhibition-deinhibition systems.

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Resumen

Se estudia la influencia de la variación del cociente NADPH/NADP sobre las actividades de las enzimas glucosa-6-fosfato deshidrogenasa y 6-fosfogluconato deshidrogenasa. Valores de este cociente de 20 y 10, son suficientes para producir una inhibición total de estas dos enzimas, respectivamente. La determinación de la concentración intracelular de algunos intermediarios metabólicos muestra que los niveles de los compuestos del ciclo de Krebs son mayores que los de los metabolitos de la ruta glicolítica. A partir de estos valores, se discute principalmente la regulación de la ruta de los fosfatos de pentosa por la relación intracelular NADPH/NADP.

Palabras clave: Ruta de los fosfatos de pentosa, Regulación, NADPH/NADP.

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