PYRUVATE KINASE FROM THE RED SKELETAL MUSCULATURE OF THE CARP (CARASSIUS CARASSIUS L.)

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SUMMARY The pyruvate kinase (E.C.2.7.1.40) from the red skeletal muscle of the carp differs markedly from the corresponding mammalian muscle enzyme by being under tight metabolite regulation. It is subject to allosteric regulation, being inhibited by ATP, l-alanine, l-phenylalanine and l-valine and activated by low concentrations of fructose-1,6-diphosphate. Fructose-1,6-diphosphate overrides all inhibitory effects of alanine, phenylalanine and ATP. The low broad pH optimum (6.8) of the carp muscle enzyme relative to other muscle pyruvate kinases is thought to be of functional significance during sustained anaerobiosis in this species.

INTRODUCTION

An important feature of fish muscle energetics is a capacity for the rapid generation of energy anaerobically during high speed cruising and sudden bursts of swimming activity (1-4). Anaerobic energy production is also important among certain species which are adapted for living in environments of low oxygen content (5). For example, European carp have been shown to be facultative anaerobes in that they apparently survive several months under conditions of near zero oxygen tension in ice-locked lakes (6). The features of glycolytic regulation in the muscles of such species enabling them to adapt both to environments of different oxygen contents and to sudden demands of muscular activity has received little attention (7). Pyruvate kinase (E.C.2.7.1.40) along with phosphofructose kinase (E.C.2.7.1.11) have been identified as important control points of glycolysis in many tissues (8). In the present study some of the regulatory properties of pyruvate kinase from carp red muscle have been described and are discussed in relation to the mode of life of this species.

MATERIALS AND METHODS

Crucian carp (Carassius carassius L.), 18-21cm long, were obtained from Stambridge Trout Fisheries, Essex between August and October 1974. They were maintained in tanks of filtered, circulated tap water at a temperature of 15±0.5°C. The fish were fed daily on a diet of chopped pigs heart. A total of 25 carp were used in these experiments. Fish were stunned by a blow to the head and killed by decapitation. Approximately 1.5g of superficial red muscle was dissected from both sides of the body, care being taken to avoid

contamination with intermediate and white fibre types (9). The muscle was cut with scissors and homogenised in an MSE homogeniser for 2 min in 3 vols of 0.01M imidazole-HCL buffer pH 7.0 at 4°C. The homogenate was centrifuged for 1 hr at 23,000g and the supernatant retained. Initial experiments were carried out on a crude purification of the pyruvate kinase achieved by ammonium sulphate fractionation between 45% and 65% saturation at 4°C. The regulatory properties of carp red muscle pyruvate kinase however were found to be extremely labile on ageing and therefore the subsequent experiments reported here were conducted directly on the high speed supernatants. These were diluted with 0.01M imidazole-HCL pH 7.0 and prepared freshly daily before each set of experiments.

Pyruvate kinase activity was measured spectrophotometrically by following the decrease in extinction at 340nm with respect to time at 22°C (10). The standard assay was conducted in a volume of 3mls under the following conditions; 100mM imidazole-HCL pH 6.8, 70mM KCL, 4mM $MgCl_2$, 2mM ADP, 1.5mM phosphoenol-pyruvate, 0.16mM NADH and excess lactate dehydrogenase. Other assay conditions where they occur are indicated in the figure legends. The reaction was started by the addition of the freshly prepared diluted tissue enzyme preparation. Blanks from which PEP[1] had been ommitted compensated for the non-specific oxidation of NADH. Enzyme activities are expressed as μmoles NADH oxidised, g wet weight muscle^{-1}, min^{-1}. All biochemicals were obtained from the Sigma Chemical Co. (London) with the exception of PEP, FDP[1], and LDH[1] (Boehringer Corp. London). Statistical analyses were carried out using analyses of variance.

RESULTS AND DISCUSSION

The saturating conditions of substrates and co-ions were found to be as follows; 70mM K^+, 4mM Mg^{2+}, 2mM ADP and 1.5mM PEP. Plots of initial velocities versus PEP and ADP concentrations were found to give hyperbolic curves as with the M_1 type PyK[1] enzyme from rat muscle (11). Michaelis constants were determined from Lineweaver-Burk plots and found to be 0.26mM and 0.34mM for PEP and ADP respectively at pH 6.8 (Fig 1). The corresponding values for rabbit skeletal muscle are 0.086mM for PEP and 0.3mM for ADP under optimal conditions (12). Thus the $Km_{(ADP)}$ is similar for both fish and mammalian skeletal muscle PyK's while $Km_{(PEP)}$ is almost 3 times higher in the case of the fish enzyme. Pyruvate kinase from carp red muscle showed a broad pH curve with an optima around pH 6.8, using imidazole buffers (Fig 2). This is in contrast to a pH optimum of 8.5 for the enzyme from rabbit skeletal muscle (13). The decrease in pH optimum shown by carp red muscle PyK relative to the corresponding enzyme from the mammal (13) and the rainbow trout (Johnston, unpublished work) may reflect the greater anaerobic potential of this muscle. Indeed it has been shown that relative to trout the carp is able to tolerate much longer and more severe periods of hypoxic exposure (14,15). Blazka (6) has reported the survival of carp under

1. Abbreviations; PyK, pyruvate kinase; PEP, phosphoenolpyruvate; FDP, D-Fructose-1,6-diphosphate; LDH, lactate dehydrogenase.

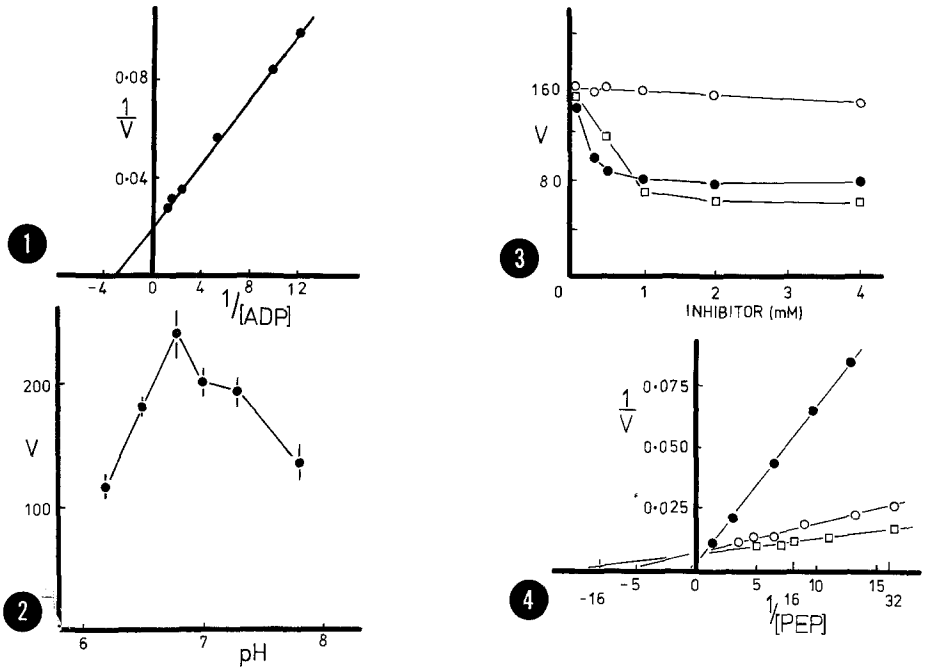

FIG. 1. A Lineweaver-Burk plot of 1/V (μmoles NADH oxidised, g. muscle^{-1}, min^{-1}) versus 1/ADP concentration (mM). Assay conditions are given in text.

FIG. 2. The effect of pH on carp red muscle pyruvate kinase activity using imidazole-HCL buffers. Assay conditions are given in the text.

FIG. 3. The effect of various concentrations of l-alanine (solid circles) and ATP (squares) on pyruvate kinase activity from the red muscle of the carp. Open circles represent the effects of 0.1mM FDP on the activity (V, μmoles NADH oxidised, gm. muscle^{-1}, min^{-1}) in the presence of various concentrations of l-alanine. Assay conditions are given in the text.

FIG. 4. A Lineweaver-Burk plot of 1/V (1/activity, see text) versus 1/PEP concentration (mM) for carp red muscle pyruvate kinase activity under various conditions; solid circles, in presence of 2mM l-alanine; open circles, in presence of 0.1mM FDP, 2mM l-alanine; squares, 0.1mM FDP. Other assay conditions are given in the text.

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conditions of almost total oxygen deprivation for periods of several months in ice-locked ponds. Under these conditions, the low broad pH optimum of carp PyK may reflect the need for a high activity of this enzyme during naturally occuring periods of extended anaerobiosis where large increases in lactic acid concentration may well result in a drop in intracellular pH.

Unlike the Pyk from mammalian species(16) the carp skeletal muscle PyK is under tight allosteric regulation by a number of metabolites. The main effects of these regulators under saturating conditions of substrates are summarised in Table 1. Carp skeletal muscle PyK also differs from the

TABLE 1

Compound Added	Absence 0.1mM FDP μmoles, g^{-1}, min^{-1}	% V_{max}	Presence 0.1mM FDP μmoles, g^{-1}, min^{-1}	% V_{max}
None	174.2 ± 6.7	100	224.7 ± 14.8	129*
2mM ATP	103.6 ± 6.5	60**	197.5 ± 17.6	113
2mM l-alanine	79.9 ± 4.3	46**	168.9 ± 12.2	97
2mM phenylalanine	91.5 ± 7.1	53**	159.4 ± 6.7	92
2mM l-valine	112.9 ± 6.0	65**	174.1 ± 14.8	100

Table 1. The effect of ATP, FDP, l-alanine, l-phenylalanine and l-valine on the activity of carp red muscle pyruvate kinase activity under saturating conditions of substrates and co-ions (V_{max}). Assay conditions are given in the text. * P. <0.05, ** P. <0.01.

corresponding rabbit and rat enzyme with respect to the effect of amino acids. L-alanine, l-phenylalanine and l-valine were all found to decrease the maximum velocity of the reaction (table 1). In the prescence of 2mM alanine the $Km_{(PEP)}$ of the reaction was found to be increased to 4mM (Fig 4). The inhibition was found to be of the mixed competitive type with respect to PEP with a K_i of 0.17mM. The inhibition of Pyk by alanine was found to be only slightly influenced by pH, being highest at neutral and acid pH's. The homologous enzyme from rat skeletal muscle has been shown to be unaffected by alanine and valine although it is inhibited by very high concentrations of phenylalanine (11). Phenylalanine inhibition is much more pronounced with carp muscle PyK. The concentration of phenylalanine which is required to give half maximal inhibition of the M_1 PyK isoenzyme of the rat is 11mM at 0.6mM PEP as opposed to only 0.5mM in the case of carp red muscle. The effects of various concentrations of alanine on carp PyK is shown in Fig 3. Inhibition by these amino acids was overcome at all concentrations by 0.1mM FDP (Fig 3). The $Km_{(PEP)}$ in the presence of 0.1mM FDP and 2mM alanine was found to be reduced to 0.18mM (Fig 4). The carp red muscle is similar with respect to its behaviour towards these amino acids to the PyK's isolated from mammalian liver (16) and turtle heart muscle (17).

Carp red muscle PyK is also inhibited by ATP as are the pyruvate kinases

from the white muscle of several other fish species (7,18). The inhibition is of the mixed competitive type with respect to PEP, 2mM ATP was found to increase the apparent $Km_{(PEP)}$ to 1.25mM. The effect of various concentrations of ATP is shown in table 1, Fig 3; 0.1mM FDP was found to override ATP inhibition.

Fructose-1,6-diphosphate (0.05-0.5mM) was found to significantly activate carp red muscle PyK activity under saturating conditions of PEP (Table 1). In addition, FDP (0.1mM) was found to lower the $Km_{(PEP)}$ 5 fold to 0.05mM, indicating a large increase in the apparent enzyme substrate affinity. Similar results have been obtained for the white muscles of several other teleosts (7,18). The strong feed-forward activation of fish muscle pyruvate kinase by FDP would allow the rapid generation of energy anaerobically as a result of a greatly increased glycolytic flux. The capacity for the rapid generation of energy in this way may well be of adaptive significance in relation to certain types of locomotary activity such as swimming at burst speeds while escaping from predators etc. In the white muscle of salmonids, for example, up to 50% of muscle glycogen can be depleted following 2 minutes swimming at high speed (1).

It is thus apparent that in contrast to the mammalian skeletal muscle isoenzyme (16) the carp red muscle pyruvate kinase is under tight metabolite regulation being allosterically modulated by certain amino acids, ATP and FDP. While the full physiological significance of these regulatory properties is unclear it may not be without significance that similar properties have been ascribed to oyster adductor muscle (19) and turtle heart muscle pyruvate kinases (17). Both of these animals have in common with the carp a capacity for facultative anaerobiosis (6,17,20). Carp red muscle like the molluscan muscles (20) has been shown to accumulate l-alanine during anaerobiosis (14). The role of anaerobic endproducts in regulating enzyme activity and carbon flow during periods of reduced oxygen availability is obviously an interesting area for future research.

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